Biochemical mechanisms of apoptosis: ordering of the biochemical events in chemical-induced apoptosis.

Thesis

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Biochemical mechanisms of apoptosis
–Ordering of the biochemical events in chemical-induced apoptosis

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by

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Abstract

Apoptosis is an important form of cell death induced by anti-cancer drugs and chemicals. The biochemical mechanisms of chemical-induced apoptosis remain, however, largely unknown, and the major biochemical events in this model of apoptosis are poorly characterised.

In this study, apoptosis, induced in human monocytic THP.1 cells by etoposide and N-tosyl-L-phenylalanyl chloromethyl ketone and in leukaemic U937 cells by etoposide, was accompanied by the release of mitochondrial cytochrome c, activation of caspases, proteolysis of poly(ADP-ribose) polymerase (PARP), DNA fragmentation, externalisation of phosphatidylserine (PS), and reduction in mitochondrial membrane potential ($\Delta\psi_m$). Time course studies demonstrated that activation of caspases occurred prior to both PS externalisation and reduction in $\Delta\psi_m$. This was further supported by the observation that the caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD.FMK) inhibited all the ultrastructural and biochemical characteristics of apoptosis except the release of cytochrome c. The execution phase of apoptosis may thus be initiated by the translocation of cytochrome c to the cytosol, where it interacts with dATP and apoptotic-protease activating factor-1, resulting in activation of the initiator caspase-9, which in turn activates effector caspases, such as -3 and -7. Cytochrome c release was a later event in non-apoptotic cell death occurring after commitment to cell death and without caspase activation. Thus its release may represent a commitment to cell death in chemical-induced apoptosis.

This study also showed that in cells triggered into apoptosis the plasma membrane changes leading to recognition and phagocytosis may be uncoupled from other features of apoptosis by using the mitochondrial inhibitors, antimycin A and oligomycin. These inhibitors blocked increased plasma membrane permeability, PS externalisation and recognition by two classes of phagocytes but not activation of caspases-3 and -7, PARP cleavage or DNA fragmentation in THP.1 cells. PS externalisation was also dissociated from caspase activation in U937 cells. Thus cell surface changes governing safe clearance of apoptotic cells may be regulated by an independent pathway to those mediated by caspases. This finding may have important consequences for attempts to manipulate cell death for therapeutic gain *in vivo*.

Finally, this study demonstrated that in chemical-induced apoptosis caspases functioned solely as executioners and activation of caspases occurred after commitment to cell death, through post-mitochondrial activation pathway(s). Z-VAD.FMK inhibited apoptosis at a stage after this commitment by blocking the post-mitochondrial activation cascade of caspases. This contrasted to its effect in death receptor-mediated apoptosis in U937 cells, where Z.VAD.FMK inhibited apoptosis prior to commitment to cell death induced by tumour necrosis factor-α.

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Publications of the results from this study:

1. Zhuang, J., Dinsdale, D. and Cohen, G.M (1998). Apoptosis, in human monocytic THP.1 cells, results in the release of cytochrome c from mitochondria prior to their ultracondensation, formation of outer membrane discontinuities and reduction in inner membrane potential. *Cell Death Differ.*, **5,** 953-962

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Chapter 1

Introduction

1.1 Defining apoptosis

Apoptosis, a term derived from a Greek word describing the dropping off of leaves from trees, is a basic form of cell death often observed when single cells are deleted in the midst of living tissues (Kerr et al., 1972). Initially, the defining characteristics of apoptosis were essentially morphological. These include loss of cell volume, dilatation of endoplasmic reticulum, compaction of cytoplasmic organelles, loss of specialised surface structures such as junctional complexes and microvilli, blebbing into a collection of membrane-bounded bodies of various sizes which are often found inside the viable neighbouring cells, as well as the distinctive condensation of chromatin into crescentic accumulation at the nuclear periphery (Kerr, 1971; Kerr et al., 1972; Wyllie et al., 1980; reviewed by Arends and Wyllie, 1991). Based on these morphological features, it is suggested that apoptosis is an active, inherently programmed phenomenon (Kerr et al., 1972). It is, however, not always the case that all the above morphological features are observed altogether in one particular cell undergoing apoptosis. In fact, time-lapse studies show that the initial cell shrinkage and fragmentation occur in a matter of minutes, whereas the phagocytosed remnants may remain recognisable within tissues for several hours, by virtue of their condensed chromatin fragments (Arends and Wyllie, 1991). Moreover, apoptosis occurs only in scattered individual cells thus making its biochemical analysis very difficult (Kerr, 1971). Perhaps due to these reasons, the incidence and biological significance of apoptosis have been appreciated only comparatively recently (Wyllie, 1988; Raff, 1992).

Apoptosis is now known to be involved in regulating cell numbers in many normal tissues and be accountable for extensive spontaneous cell loss in malignant neoplasms (reviewed by Wyllie, 1992 and 1993). It is also implicated in both physiological involution and atrophy of various tissues and organs (Kerr, 1971; Kerr et al., 1972). In the ontogeny of the immune system apoptosis occurs in deletion of autoreactive T lymphocyte clones during thymic maturation (Kisielow et al., 1988; Smith et al., 1989) and in B lymphocyte deletion in germinal centres (Liu et al., 1989). Apoptosis also plays a vital role in many normal embryonic processes of animal development such as the formation of the lumina of tubular structures, the development of limbs, and involution of phylogenetic vestiges (reviewed by Clarke, 1990; Jacobson et al., 1997).

Apoptosis, a mode of cell death distinct from necrosis, is often interchangeably referred to as programmed cell death. Programmed cell death was well recognised in the 19th century and was a focus of interest in early developmental biology (reviewed by Clarke and Clarke, 1996; Lockshin, 1997). It has an obvious meaning when applied to cell deletion occurring as part of developmental processes, such as rearrangement of cells in forming the avian limb as mentioned above, and apoptosis appears to be the major form of cell death. However, there are many other instances in which programmed cell death occurs by a morphology other than apoptosis, notably cell death in interdigital membranes of the feet of chick embryo in forming the interdigital clefts (reviewed by Clarke, 1990; Majno and Joris, 1995; Schwartz, 1995). Likewise, apoptosis can occur in cells when challenged with many unphysiological stumuli including some toxic chemicals and ionising radiation (reviewed by Arends and Wyllie, 1991; Trump et al., 1997), suggesting that at least in some situations apoptosis is not programmed but is the cells' response to changes in their local environment . Furthermore, there is a conceptual confusion due to the fact that two different programs are involved in apoptosis, one is to carry out the death process while the other is to trigger the activation of this death process, and that both programs appear to be genetically regulated. Two programs are thus defined by two different concepts. The genetic program of programmed cell death is a clock specifying the time for cell death to occur, whereas the genetic program of apoptosis specifies the machinery to execute death process which eventually brings about the morphological features of apoptosis. Therefore, apoptosis refers to a morphology while programmed cell death describes a process. For these reasons, the term apoptosis is used throughout this thesis as defined by the morphological criteria.

1.2 Morphological and biochemical features

As described earlier, apoptosis involves a distinct morphological process which may occur in three discrete stages (Kerr et al., 1972; Wyllie, 1988; Arends and Wyllie, 1991). In the first, there is reduction in nuclear size, condensation of chromatin, loss of cell to cell contact and dilatation of the endoplasmic reticulum. Cell membranes are still intact as cells within tissues do not evoke inflammatory reaction. The second stage mainly comprises breaking up of the cell into a number of membrane-bound "apoptotic bodies", some containing nuclear fragments, and most of the apoptotic bodies are phagocytosed by neighbouring cells or macrophages. In the third stage, there is progressive degeneration of residual nuclear and cytoplasmic structures. Cell membranes disappear and organelles

become unrecognisable. The majority of apoptotic cells seen under the light microscope are in fact in the latter two stages. It is estimated that within tissues the whole process is completed in less than ten hours (Arends and Wyllie, 1991). This relatively short period indicates that high rates of apoptosis produce only small increases in the proportion of apoptotic cells recognisable in tissue sections. Swift clearance of apoptotic cells also protects surrounding tissues from exposure to injurious contents leaking from dying cells, which is inevitable in accidental cell death culminating in necrosis.

The characteristic morphologic condensation of chromatin in apoptosis is often associated with DNA fragmentation. Initially , DNA is cleaved to 200-300 and 30-50 kilobase pair-sized fragments, which in terms of higher-order chromatin structure may represent loops and rosettes of DNA (Brown et al., 1993; Cohen et al., 1994). The large kilobase pair-sized fragments are then further degraded to form internucleosomal DNA fragments of 180-200 basepair in length. These internucleosomal fragments, demonstrated as a DNA ladder by agarose gel electrophoresis, were originally considered as a biochemical hallmark of apoptosis (Wyllie, 1980). The formation of internucleosome-sized DNA fragments is now considered as a late event in apoptosis, which may be dissociated from early more critical changes (G. Cohen et al., 1992).

Another biochemical feature associated with apoptosis is the degradation of a set of cellular proteins. Proteolysis of nuclear proteins such as lamins (Kaufmann, 1989; Oberhammer et al., 1994), poly (ADP-ribose) polymerase (PARP) (Kaufmann, 1989; Kaufmann et al., 1993), DNA-dependent protein kinase (DNA-PK) (Casciola-Rosen et al., 1995; Song et al., 1996) and U1 small ribonucleoprotein (U1-70 kDa) (Casciola-Rosen et al., 1994; Tewari et al., 1995a) has consistently been observed in cells undergoing apoptosis induced by diverse apoptotic stimuli in a variety of cell systems. Although the precise biological significance of these proteolytic cleavages and their relationship with the ensuing apoptotic morphology are yet unknown, it is believed that degradation of these nuclear proteins contributes to the collapse of the nucleus and condensation of the chromatin. Of particular interest is the DNA repair enzyme PARP (Satoh and Lindahl, 1992) which is cleaved early during apoptosis in a number of cell systems and its cleavage is thus proposed as another biochemical marker of apoptosis (Kaufmann et al., 1993).

1.3 Apoptosis and diseases

Apoptosis is a fundamental form of cell death which plays a major role in the development and homeostasis of multicellular organisms, as described earlier. It is also imperative for host survival as it provides a simple way to eliminate misplaced, damaged or infected cells. Disturbances in apoptosis have therefore been implicated in pathogenethesis of a variety of human diseases including cancer, acquired immunodeficiency syndrome (AIDS), autoimmune diseases and some neurodegenerative disorders (reviewed by Thompson, 1995). These diseases either occur as a result of increased apoptosis or are associated with the inhibition of apoptosis, as shall be described below.

1.3.1 Diseases where apoptosis is inhibited

1.3.1.1 Cancer

The observation that most, if not all, mammalian nucleated cells undergo apoptosis when deprived of exogenous signals and /or intercellular contacts has led to the notion that survival of a cell depends on the presence of signals produced by other cells, and in addition, its survival may require continuous suppression of apoptotic process (reviewed by Raff, 1992). Dependence of normal cells on environment-specific factors to maintain their viability may serve to prevent normal cells from surviving in non-physiological sites. Metastatic tumour cells have, however, circumvented this homeostatic mechanism and can survive at sites distinct from the tissues in which they arise. Consistent with the early suggestion that hyperplasia might result from decreased apoptosis rather than increased mitosis (Kerr et al., 1972), cells from a wide variety of human malignancies show a decreased ability to undergo apoptosis in response to several physiological stimuli (Hoffman and Liebermann, 1994). Recent studies on the molecular bases for the decreased apoptosis in tumour cells have suggested the involvement of several critical genes in the regulation of apoptosis (reviewed by Wyllie, 1992; Reed, 1994; Thompson, 1995).

The gene *bcl-2* was first discovered because of its involvement in B cell malignancies, where translocations between chromosomes 14 and 18 activates the gene in the majority of follicular non-Hodgkin's lymphomas (Tsujimoto et al., 1985). Though originally viewed as an oncogene, Bcl-2 protein is found to have little ability to promote cell cycle or cell proliferation (reviewed by Adams and Cory, 1998). Studies using immature B

cells that are dependent on the cytokine, interleukin-3, for their growth and survival in culture show that stable transfer of *bcl-2* gene permits prolonged cell survival in the absence of the cytokine, but with no effect on cell proliferation (Vaux et al., 1988). Further studies demonstrate that Bcl-2 is capable of both promoting B cell survival and follicular lymphoproliferation in transgenic mice (McDonnell et al., 1989) and blocking apoptosis in growth factor-deprived haemopoietic cell lines (Hockenbery et al., 1990; Nunez et al., 1990). Moreover, in contrast to the prolonged cell survival and anti-apoptotic potential observed when elevations in the levels of Bcl-2 protein are achieved through gene transfer methods, antisense-mediated reductions in Bcl-2 expression have been shown to accelerate apoptosis following growth factor withdrawal (Reed et al., 1990a). In addition, over-expression of Bcl-2 can prevent or markedly reduce apoptosis induced by a wide variety of stimuli (reviewed by Nunez and Clarke, 1994; Kroemer, 1997; Adams and Cory, 1998). In all cases where examined, Bcl-2 appears to block a relatively early event associated with apoptotic cell death in that none of the characteristic morphological changes such as cell shrinkage, chromatin condensation, and nuclear fragmentation occurred, and DNA degradation was markedly reduced or prevented (reviewed by Reed, 1994). This suggests that Bcl-2 blocks a final common pathway leading to apoptotic cell death, although the mechanisms underlying the inhibition are not fully understood yet. The observation that Bcl-2 blocks apoptosis induced by many chemotherapeutic drugs as well as γ-irradiation (Miyashita and Reed, 1992; Strasser et al., 1994) may provide some clues, as these stimuli all induce DNA damage either directly or indirectly. The protection afforded by Bcl-2 does not involve reductions in drug-induced damage to DNA, increased rates of DNA repair, or changes in cell cycle kinetics (Fisher et al., 1993; Walton et al., 1993). Thus, Bcl-2 appears to act downstream of all of these events, perhaps by preventing the occurrence of damaged DNA from being translated into a signal for activation of the apoptotic program, or by blocking the action of individual components of the program after its activation or both.

There is increasing evidence that the tumour suppresser gene *p53* may be involved in this pathway. The protein product of the *p53* gene has been implicated in maintaining genomic stability, and in response to drug-induced DNA damage two cellular response to p53 activation are well described, i.e. growth arrest (in cell cycle stages G_1 and G_2) and apoptosis (reviewed by Levine, 1997; Evan and Littlewood, 1998). It is thus tempting to speculate that a functional connection between p53 and Bcl-2 may exist. It has indeed

been shown that over-expression of Bcl-2 blocks p53-induced apoptosis in several cell systems (Wang et al., 1993; Chiou et al., 1994). However, this is by no means to suggest that p53 is the only mediator in regulation of apoptosis by Bcl-2 since studies using *p53* knock-out mice have shown that p53 is required for induction of apoptosis by some stimuli such as γ-irradiation and the anti-cancer drug etoposide but not by glucocorticoids and Ca^{++}-ionophores in thymocytes (Lowe et al., 1993; Clarke et al., 1993), and yet Bcl-2 is very effective in blocking glucocorticoids and Ca^{++}-ionophore-induced cell death (Sentman et al., 1991; Miyashita and Reed, 1992).

Studies investigating other genes required for apoptosis in the absence of p53 suggest that some oncogenes are involved in regulating the induction of apoptosis, in cooperating with Bcl-2. c-Myc is a key regulator of cell growth and differentiation and Myc oncoproteins are commonly activated in human cancer (reviewed by Packam and Cleveland, 1995; Evan and Littlewood, 1998). It has been shown that expression of *c-myc* induces apoptosis in several systems (Evan et al., 1992; Shi et al. 1992). Gene transfer experiments have shown that Bcl-2 blocks apoptosis induced by *c-myc* (Bissonnette et al., 1992; Fanidi et al., 1992), indicating that Bcl-2 suppresses apoptotic signals that occur with transformation. It also suggests that, as c-Myc stimulates both mitogenesis and apoptosis, concomitant expression of Bcl-2 can attenuate the apoptotic influence of *c-myc*, thus reinforcing the proliferative effects of *c-myc* and leading to a further selective growth advantage of tumour cells. Bcl-2 has also been shown to co-operate with members of the *ras* oncogene family in malignant transformation of rat embryonic fibroblasts (Reed et al., 1990b). Consistent with the above experimental observations, the presence of either the Bcl-2 protein or *bcl-2* gene rearrangement has clinically been associated with a poor prognosis in patients with prostatic cancer (McDonnell et al., 1992), non-Hogkin's lymphomas (Yunis et al., 1989), and acute myeloid leukaemia (Campos et al., 1993).

1.3.1.2 Autoimmune disorders

Apoptosis is responsible for the removal of potentially autoreactive lymphocytes during development as well as for the removal of excess cells after the completion of an immune response (reviewed by Golstein et al., 1991; J. Cohen et al., 1992). Disregulation of apoptosis has been implicated in the aetiology of autoimmune diseases. Studies in murine models have shown that one molecule critical in regulating apoptosis in lymphocytes involves the cell surface receptor CD95 (also known as Fas or Apo-1), a member of the

tumour necrosis factor (TNF) receptor family (reviewed by Nagata and Golstein, 1995; Nagata, 1997). Stimulation of CD95 on activated lymphocytes can induce apoptosis. Two forms of hereditary autoimmune diseases have been attributed to alterations in CD95-mediated apoptosis (Watanabe-Fukunaga et al., 1992; Suda et al., 1993). Mice carrying homozygous mutations in *lpr* (lymphoproliferation) develop a fatal autoimmune disorder by 6 months of age, resembling human systemic lupus erythematosus, whereas mutations in *gld* (generalised lymphoproliferative disease) also result in a similar illness (Cohen and Eisenberg, 1991). Genetic and molecular analyses of *lpr* and *gld* mutations have shown that they are loss-of-function mutations in genes encoding CD95 and CD95 ligand, respectively (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994). A lupus-like autoimmune disease has also been reported in transgenic mice constitutively over-expressing Bcl-2 in their B cells (Strasser et al., 1991).

Children carrying a defect in the CD95 gene have also been identified with most of these patients carrying a heterozygous mutation (Fisher et al., 1995; Rieux-Laucat et al., 1995). T cells from the patients do not undergo apoptosis upon activation. The patients show phenotypes (autoimmune lymphoproliferative syndrome) that bear marked similarity to those of *lpr* mice, including lymphadenopathy, splenomegaly, and hypergammaglobulinemia. Some patients show autoimmune conditions such as haemolytic anaemia, thrombocytopenia, and neutropenia by producing autoantibodies against red blood cells and platelets. It should be pointed out that the studies into the role of apoptosis in the development of autoimmune diseases in humans are still in an early stage although alterations in the susceptibility of lymphocytes to die by apoptosis *in vitro* have been reported in several other diseases (reviewed by Thompson, 1995).

1.3.1.3 Viral infections

Apoptosis can occur in cells infected by viruses (Levine et al., 1993) and this may serve as a cellular defence mechanism to prevent viral multiplication. In addition, cytotoxic T cells act to prevent viral spread by recognising and killing cells exhibiting viral peptides associated with cell surface major histocompatibility complex (MHC) class I molecules (Henkart, 1994). Cytotoxic T cells induce apoptosis by either activating the death receptor, e.g. CD95, on the surface of the target cell or producing proteases, e.g. granzyme B, which activate the cell death program from within the cells (reviewed by J. Cohen et al., 1992; Nagata and Golstein, 1995; Froelich et al., 1998). Several studies

have shown, however, that many viruses circumvent the host defences by interfering with the natural apoptotic processes that are activated in the host cells. For example, the adenovirus E1B 19 kDa protein that is required to establish an effective adenoviral infection blocks apoptosis directly and its function can be replaced in adenovirus by Bcl-2 (Rao et al., 1992; Boyd et al., 1994). Other viral genes have also been reported to inhibit apoptosis. Cowpox virus effectively inhibits apoptosis in a number of different systems (Miura et al., 1993 and 1995; Chinnaiyan et al., 1996; Srinivasula et al., 1996a). One of the viral genes necessary for this inhibition, *crmA* (a cytokine response modifier gene), encodes a serpin that effectively inhibits apoptosis-specific proteases (Ray et al., 1992), now known as caspases (see below). CrmA has also been shown to inhibit the development of an inflammatory response in virally infected cells (Gagliardini et al., 1994). The protein products of baculovirus *p35* and the inhibitor of apoptosis (*IAP*) genes can inhibit apoptosis induced by a wide variety of stimuli (Clem et al., 1991 and 1994; Liston et al., 1996). Further studies show that both P35 and IAP proteins act as specific inhibitors of caspases (Bump et al., 1995; Hawkins et al., 1996; Deveraux et al., 1997; Roy et al., 1997). The prevention of apoptosis is also vital for the establishment of viral latency as indicated by an observation that Epstein-Barr virus (EBV) enhances the survival of infected B cells (Gregory et al., 1991). The survival effect may be conferred by expression of a single EBV latent protein LMP-1, which specifically up-regulates the expression of Bcl-2, thus providing a survival advantage to latently infected cells (Henderson et al., 1991).

1.3.2 Diseases associated with excessive apoptosis

Excessive apoptosis can result from both intra-and extra-cellular changes that enhance the accretion of signals that induce apoptosis or that decrease the threshold of the induction of apoptosis. Several clinical conditions are believed to be associated with the increase in apoptosis.

1.3.2.1 AIDS

Lymphopenia and immunodeficiency in patients with AIDS, induced by the human immunodeficiency virus (HIV), may be linked to an enhanced susceptibility of the $CD4^+$ helper T lymphocyte population to death by apoptosis (Ameisen and Capron, 1991; Gougeon and Montagnier, 1993). *In vitro* activation of $CD4^+$ T cells from asymptomatic HIV-infected individuals by mitogens induces apoptosis (Groux et al., 1992). In addition,

specific $CD4^+$ T cell death is observed upon activation of T cells by the MHC class II-dependent bacterial superantigens. Although the mechanism underlying the increased susceptibility is not fully understood yet, the binding of CD4 receptor by the glycoprotein gp120 of the virus is believed to prime normal $CD4^+$ T cells for apoptosis in response to subsequent T cell receptor stimulation. Both $CD4^+$ T cells from normal and from HIV-infected individuals have indeed been shown to undergo apoptosis *in vitro* if cell surface CD4 is crosslinked before engagement of T cell receptors (Newell et al., 1990; Banda et al., 1993). Thus, it is possible that gp120 promotes apoptosis by its interaction with CD4. It has also been suggested that interference of signal transduction by HIV can result in the induction of apoptosis in T lymphocytes, perhaps by inducing oxidative stress or an abortive cell cycle (reviewed by Orrenius, 1995). As $CD4^+$ T cells play an important role in establishing protective immunity against a wide variety of viral infections, development of chronic HIV infection may therefore depend on virally mediated depletion of $CD4^+$ T cells and the concomitant loss of a protective cell-mediated immune response in host.

1.3.2.2 Neurodegenerative disorders

Many neurological diseases are characterised by the gradual loss of specific sets of neurons (reviewed by Isacson, 1993). These disorders includes Alzheimer's disease, Parkinson's disease, amyotrophic lacteral sclerosis (ALS), retinitis pigmentosa, spinal muscular atrophy, and several forms of cerebellar degeneration. The cell loss in these diseases does not provoke an inflammatory response and appears to be accomplished by apoptosis (reviewed by Desmukh and Johnson, 1997). Oxidative stress, calcium toxicity, mitochondrial defects, excitatory toxicity, and deficiency of survival factors have all been speculated to contribute to the pathogenesis of these diseases by increasing susceptibility of neurons to apoptosis (reviewed by Choi, 1992; Orrenius, 1995). In support of this view, the anti-apoptotic Bcl-2 protein has been shown to protect a central neural cell line from apoptosis induced by diverse stimuli (Zhong et al., 1993; Offen et al., 1997).

Most cases of early-onset familial Alzheimer's disease are caused by mutations in genes encoding the presenilin 1 and 2 proteins (reviewed by Hardy, 1997). The resultant mutant proteins have been shown to induce an increased production of β-amyloid, a peptide progressively accumulated in plaques in patients with Alzheimer's disease (Scheuner et al., 1996; Duff et al., 1996). *In vitro* studies have shown that in neurons apoptosis is

induced by β-amyloid (Loo et al., 1993), suggesting that excessive apoptosis may occur *in vivo* leading to gradual depletion of neurons and subsequent development of the disease. The antioxidant and free radical scavenger vitamin E inhibits β-amyloid-induced cell death (Behl et al., 1992), indicating the involvement of reactive oxygen species (ROS) in apoptosis. In addition, evidence from human brain studies and animal transgenic experiments supports a role of apoptosis in Alzheimer's disease (reviewed by Dragunow et al., 1997), although clinical significance of these findings still remains to be further substantiated.

Parkinson's disease is another form of neurodegenerative disorder characterised by progressive cell loss confined mostly to dopamine neurons of the substantia nigra (reviewed by Burke and Kholodilov, 1998). Characterisation of cell death reveals morphological and biochemical features of apoptosis in both nigral neurons of patients with Parkinson's disease (Anglade et al., 1997) and neural cell lines (Ziv et al., 1994; Walkinshaw and Waters, 1995). Cells over-expressing Bcl-2 show a marked resistance to cell death (Offen et al., 1997), suggesting that apoptosis may contribute to the development of the disease. The accumulation of ROS generated during altered metabolism of the natural neurotransmitter dopamine may be responsible for the increased apoptosis in neurons as the treatment with an antioxidant inhibits the cell death (Ziv et al., 1994).

The spinal muscular atrophies (SMAs), characterised by spinal cord motor neuron depletion, are a group of recessive neurodegenerative disorders of childhood. One of the genes linked to these disorders has been isolated recently, which encodes neuronal apoptosis inhibitory protein (NAIP), a homologue to the baculovirus inhibitor of apoptosis protein (Roy et al., 1995; Liston et al., 1996). It has been shown that the gene is partly deleted in individuals with SMAs, suggesting that the lack of suppression of apoptosis by NAIP results in motor neurons more susceptible to apoptosis.

Retinal degeneration associated with retinitis pigmentosa, a condition leading to progressive blindness, may result from mutations in any one of the three photoreceptor-specific genes encoding rhodopsin, the β subunit of cyclic guanosine monophosphate phosphodiesterase, and peripherin, respectively (Portera-Cailliau et al., 1994). All three mutations lead to photoreceptor apoptosis in animal models (Chang et al., 1993; Portera-

Cailliau et al., 1994). Although the initiating mechanisms of apoptosis associated with these conditions are not known yet, accumulation of the mutant protein with dysfunctional structure may result in an increased apoptosis (Rao et al., 1994).

1.3.2.3 Blood cell disorders

Mature blood cells are continuously being produced from haemotopoietic stem cells in the bone marrow. Several haematological diseases are, however, associated with a decreased production of blood cells. These disorders include anaemia resulting from chronic diseases, aplastic anaemia, chronic neutropenia, and the myelodysplastic syndromes, which are associated with increased apoptotic cell death within the bone marrow (Yuan et al., 1993a; reviewed by Yoshida, 1993). The diseases could result from the activation of genes promoting apoptosis, deficiencies in haematopoietic survival factors or the direct effects of toxins. In support of this view, several haematopoietic growth factors have been shown to increase the production of individual types of blood cells (reviewed by Fleischman, 1993). For instance, erythropoietin is used to increase red blood cell production in patients with anaemia caused by renal failure and other chronic conditions. Granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage CSF, and granulocyte CSF have all been used to promote the recovery of macrophages and granulocytes following chemotherapy for cancer (reviewed by Thompson, 1995).

1.3.2.4 Myocardial infarction and stroke

Myocardial infarction and stroke are two common disorders all caused by an acute loss of blood flow (ischemia). In both disorders, cells within the central area of ischemia appear to die rapidly resulting in necrosis. Cells outside of the central ischemic region, however, die over a prolonged time period, morphologically by apoptosis (Cohen, 1993). Ischemia of both neurons and cardiac myocytes in culture result in the induction of apoptosis (Rosenbaun et al., 1994; Tanaka et al., 1994). Agents known to be inhibitors of apoptosis *in vitro* have been shown to limit infarct size *in vivo*. For example, use of apoptosis-specific protease inhibitors reduces ischemic and excitotoxic neuronal damage in mice (Hara et al., 1997). Thus, it is likely that apoptosis plays an important role in the development of these diseases.

1.4 Evolutionarily conserved mechanism of apoptosis

The observation that apoptosis is often accompanied by the distinct morphological changes has led to the idea that there must be a set of biochemical events that characterise the apoptotic program. Furthermore, there is increasing evidence that apoptosis occurs by a mechanism that has been at least partially conserved throughout animal evolution. Indeed, much of our current knowledge about the molecular basis of the death program came initially from genetic studies in the nematode *Caenorhabditis elegans*. During the development of the *C. elegans* hermaphrodite, 1090 cells are generated, of which 131 undergo apoptosis as a result of programmed cell death (reviewed by Ellis et al., 1991). Genetic analyses of *C. elegans* have identified genes that function in apoptosis and ordered these genes into a genetic pathway, which led to the finding that similar genes operate during apoptosis in other organisms, including mammals.

1.4.1 Genes required for cell death during the development of the *C. elegans*

Cell death by apoptosis in the development of *C. elegans* may be divided into four stages, including the decision of whether a particular cell will die, the death process of the cell, the engulfment of the dead cell by phagocytes, and the degradation of the engulfed corpse (reviewed by Steller, 1995). A variety of single-gene mutations that have specific effects in each of these stages during cell death have been identified, which define a number of genes required not only to cause cell death but also to protect cells from dying (reviewed by Metzstein et al., 1998).

The activity of the gene *ced-9* (*ced*, cell death defective) protects most, if not all, cells from undergoing programmed cell death during *C. elegans* development. A dominant gain-of-function mutation in *ced-9* suppresses all normal programmed cell death, whereas recessive loss-of-function mutations in *ced-9* cause many normally living cells to undergo apoptosis and are lethal to *C. elegans* (Hengartner et al., 1992). These observations suggest that the protein product of *ced-9* acts as a gatekeeper to prevent cells from activating the cell death program.

Three other *C. elegans* genes, *egl-1* (*egl*, egg-laying defective), *ced-4* and *ced-3* seem to be required for all somatic cell death to occur. Loss-of-function mutations in *egl-1*, *ced-4* or *ced-3* lead to the survival of the cells that normally undergo apoptosis during wild-type development (Ellis and Horvitz, 1986; Conradt and Horvitz, 1998). The gene *egl-1* encodes a protein that interacts with Ced-9 protein, and gain-of-function mutations in *egl-*

1 cause the two hermaphrodite-specific neurons (HSNs), which are required for egg laying, to undergo apoptosis inappropriately (Conradt and Horvitz, 1998). A loss-of-function mutation in *egl-1* prevents not only the ectopic death of the HSNs but also apoptosis in most cells otherwise undergoing programmed cell death. On the other hand, recessive loss-of-function mutations in *ced-3* and *ced-4* prevent all 131 cells from undergoing programmed cell death during *C. elegans* hermaphrodite development (reviewed by Yuan, 1996). Such mutations can also prevent the excess cell death and lethality in *ced-9* loss-of-function mutant animals (Hengartner et al., 1992), indicating that *ced-3* and *ced-4* function genetically downstream of *ced-9*. In addition, mutations in either *ced-3* or *ced-4* are sufficient to eliminate all programmed cell death, suggesting that the functions of these two genes are not redundant (Ellis and Horvitz, 1986). Thus, the Ced-3 and Ced-4 proteins may well represent the machinery that carry out the execution of the cell death program within the dying cells.

Further genetic studies suggest that *ced-4* acts upstream of *ced-3* since cell death induced by *ced-4* over-expression is greatly reduced in the absence of activity of cell death protease Ced-3 (Shaham and Horvitz, 1996). It has recently been shown that interaction of Ced-4 with Ced-3 results in the activation of Ced-3 and the initiation of cell death (Seshagiri and Miller, 1997; Chinnaiyan et al., 1997a and b). It has also been shown that Ced-9 protein functions to negatively regulate the cell death by binding to Ced-4 protein (Chinnaiyan et al., 1997b; Spector et al., 1997; Wu et al., 1997), thus preventing the activation of Ced-3 (Hengartner, 1997). However, these observations do not exclude the possibility that *ced-9* may also employ other mechanism(s) in suppressing apoptosis apart from inhibiting the activation of *ced-4* and *ced-3*. Indeed, recent studies show that Ced-9 can also directly inhibit the activity of Ced-3 protease in *C. elegans* (Xue and Horvitz, 1997). By contrast, loss of *egl-1* function, while preventing somatic cell death as does loss of *ced-4* or *ced-3* function, does not prevent the cell death and lethality caused by loss of ced-9 function (Conradt and Horvitz, 1998), suggesting that *egl-1* may act genetically upstream of *ced-9*. As Egl-1 protein binds to the Ced-9 protein, it has been proposed that Egl-1 acts by releasing Ced-4 from Ced-9 thus functioning as a negative regulator of Ced-9 (Metzstein et al., 1998).

1.4.2 Mammalian homologues of the cell death genes

The molecular characterisation of *C. elegans* cell death genes has significantly contributed to the understanding of some of the biochemical mechanisms underlying apoptosis in mammals. The discovery that *ced-3* encodes a protease homologous to mammalian interleukin-1β converting enzyme (ICE) (Yuan et al., 1993b) was instrumental in identifying a role of mammalian Ced-3-like proteases in the regulation of apoptosis. To date, there are at least 13 mammalian Ced-3/ICE-related proteases identified (reviewed by Thornberry and Lazebnik, 1998), and a unified nomenclature for these proteases has been established after the name caspase, with the "c" denoting a cysteine protease and the "aspase" referring to the unique ability of these proteases to cleave specifically after an aspartic acid residue (Alnemri et al., 1996). Another mammalian protein related to the Ced-4 protein, Apaf-1 (Apaf, apoptotic protease activating factor), appears to be critically involved in a pathway leading to the activation of some of these caspases (Zou et al., 1997, see below). Cloning of the *ced-9* gene shows that it encodes a 280-amino acid protein containing sequence and structural similarities to the mammalian Bcl-2 protein (Hengartner and Horvitz, 1994). This discovery, together with the findings that human Bcl-2 can block programmed cell death in *C. elegans*, thus functionally replacing Ced-9 (Vaux et al., 1992), suggests that at least some of molecular mechanisms of apoptosis are evolutionarily conserved. In addition, Egl-1 has functional and molecular similarity to the "BH3-only" (BH, Bcl-2 homology domain) subfamily of Bcl-2-like proteins (reviewed by Adams and Cory, 1998; Metzstein et al., 1998). BH3-only proteins have been shown to induce apoptosis when over-expressed in mammalian cell systems (Conradt and Horvitz, 1998).

The structural and functional similarities between *C. elegans* Ced-9, Ced-4, Ced-3 and Egl-1 proteins and their respective mammalian counterparts strongly suggest an evolutionary conservation of a genetic pathway of apoptosis in multicellular organisms. It is likely that additional mammalian apoptosis-regulating genes will be identified that may share similarities with other *C. elegans* cell death genes. However, the situations in mammals are much more complicated, for instances, instead of *ced-9, ced-4, ced-3* and *egl-1*, there are already many more *bcl-2*-related genes (Adams and Cory, 1998) and many more *caspase*-related genes (Thornberry and Lazebnik, 1998) reported in mammalian cells. Furthermore, it is also expected that some other critical biochemical events may be involved in mammalian cell death whereas they may not be important in *C. elegans* cell death, as will be described below.

1.5 Major biochemical events of apoptosis in mammalian cells

The evolutionary conservation of the molecular machinery responsible for apoptosis, as described above, also suggests that apoptosis occurs through the activation of an intrinsic cell suicide program. The basic machinery to execute apoptosis appears to be constitutively present in almost all mammalian cells, but the activation of the cell death program may be carefully regulated by many different signals of both intra- and extra-cellular origin (reviewed by Steller, 1995; Wyllie, 1995). These diverse signals may function to either suppress or promote the activation of the death program. Apoptotic cell death thus occurs in two phases, an initial commitment phase followed by the execution phase (reviewed by Earnshaw, 1995). Upon receiving a signal which can be either external or internal, the cell responds to it. The length of time for decision making between life and death varies enormously, thus making it extremely difficult for the biochemical characterisation of the molecular events involved in this phase. Once cells become committed to death, there occurs a transition to an execution phase. This execution phase, characterised by a series of distinct morphological and biochemical changes of apoptotic phenotype, is brief and decisive (Earnshaw, 1995). As a result, the cell has ceased its existence as an integral unit.

The understanding of the elaborate sequence of biochemical events during the execution phase of apoptosis has so far been dominated by three major observations. Firstly, apoptosis is the outcome of a systematic dismantling cascade of key cellular components and activation of caspases appears to be primarily responsible for this to occur. Secondly, many important apoptosis-related molecules, e.g. Bcl-2 and cytochrome c, are localised in mitochondria. Alterations in mitochondrial function and structure also occur during the induction of apoptosis, thus suggesting that mitochondria co-ordinate the apoptotic process in execution phase. Finally, both activation of caspases and mitochondrial changes have been implicated in resulting in the plasma membrane changes in cells undergoing apoptosis, which lead to the recognition and phagocytosis thus completing the elimination of dead cells. However, many questions remain unresolved, for example, how these events are regulated and what the temporal relationships between these events are and so on. Below is a literature review on the background of these individual events and their biological significance in apoptosis.

1.5.1 Activation of caspases

Apoptosis is accompanied by a cascade of proteolysis of a set of cellular proteins resulting in disassembly of the cell. This cascade is centred on the activation of caspases, which is triggered in response to proapoptotic signals through several pathways. Thus caspases represent a proteolytic system, a central component of the execution machinery of apoptosis within the cell.

1.5.1.1 Biochemical properties of caspases

With the cloning of *ced-3* in *C. elegans* comes the observation that it encodes a protease homologous to the mammalian ICE (now renamed as caspase-1) (Alnemri et al., 1996). Together with the finding that apoptosis can be induced in mammalian cells by transient expression of caspase-1 (Miura et al., 1993), these studies demonstrate for the first time that there exists a conserved family of proteases acting as a critical component of the cell death machinery. Thus far there are 13 members of caspase family being identified (Table 1). In addition to their sequence similarity to Ced-3, the members of caspase family of proteases have several general properties. As indicated by their names, caspases are cysteine proteases, each containing a conserved QACRG pentapeptide active-site

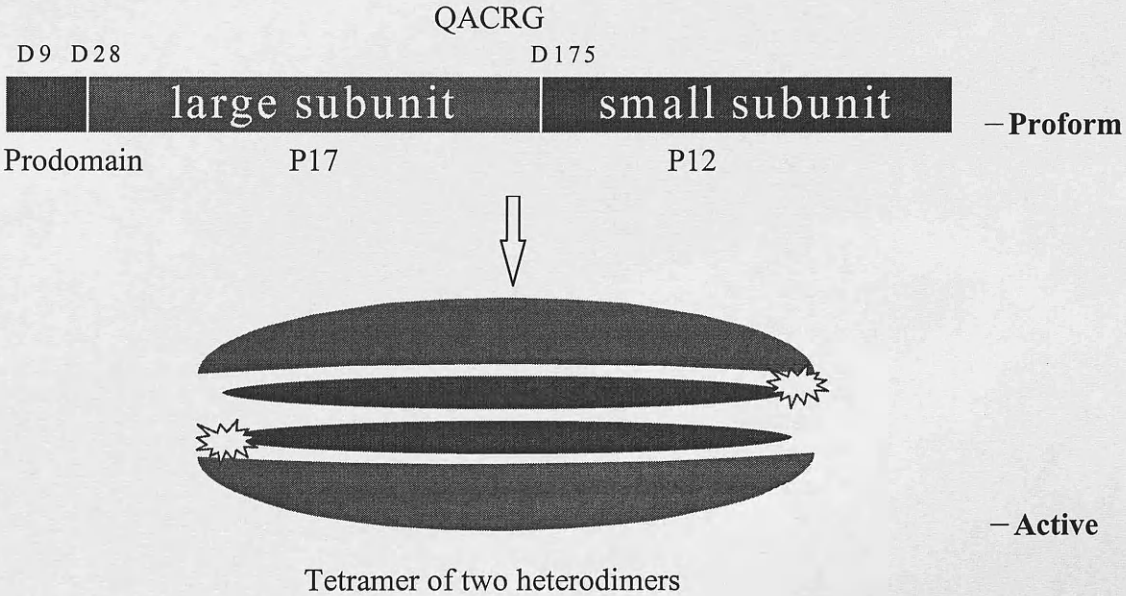


Figure 1. Proposed structures of pro- and active-caspase-3

In common with other caspases, caspase-3 is synthesised as an inactive proenzyme and upon activation, is cleaved at specific aspartate residues, generating a large and a small subunit, which together form an active enzyme in the form of a heterotetramer containing two large and two small subunits. The amino-terminal prodomain, which is highly variable in length and sequence, is involved in regulating caspase activation. The flashing points refer to the active sites of the protease.

motif (reviewed by Nicholson, 1996; Cohen, 1997). Caspases are synthesised as catalytically inactive proenzymes composed of a variable length amino-terminal prodomain, a large subunit (~20 kDa) and a small subunit (~10 kDa) (Fig. 1). Activation of caspases requires proteolytic cleavage of the proenzyme at specific aspartate residues separating these three domains, thereby resulting in the removal of the prodomain and formation of a heterodimer containing one large and one small subunit. Crystallographic analyses have shown that the active caspase is a tetramer containing two such heterodimers, with two catalytic sites that appear to function independently (Walker et al., 1994; Wilson et al., 1994; Rotonda et al., 1996). Within each catalytic domain, the large and small subunits are closely associated, with both contributing residues necessary for substrate binding and catalysis. The amino-terminal prodomain, which is highly variable in length (from 23 to 216 amino acids) and sequence, is believed to regulate the activation of these enzymes (reviewed by Cryns and Yuan, 1998). Caspases are among the most specific of proteases with an unusual and absolute requirement for cleavage after aspartic acid (Thornberry et al., 1992; Thornberry and Molineaux, 1995). This distinctive substrate specificity, coupled with their necessary proteolytic activation at aspartic residues, suggests that autoproteolysis exists in caspases, which may be responsible for self-activation and/or activation of other caspases. In addition, efficient catalysis also requires four amino acids amino-terminal to the cleavage site of the substrate to serve as recognition motif. However, this preferred tetrapeptide recognition motif differs significantly among individual caspases, especially the amino acid at P4 position of the substrate (Thornberry et al., 1997). This may not only explain the diversity of their biological functions, but also suggest that caspases cleave only a set of specific substrates with optimal tetrapeptide sequences. The stringent specificity of caspases is consistent with the observation that apoptosis is accompanied by the proteolysis of a select set of cellular proteins, but not a random protein digestion. Recent studies using a positional scanning substrate combinatorial library or synthetic peptides divide caspases into three subgroups based on their substrate preferences (Talanian et al., 1997; Thornberry et al., 1997). The first group comprises of caspases-1, -4, and -5 which prefer bulky hydrophobic residues in the P4 position and have the optimal substrate cleavage sequence WEXD. The second group consists of caspases-2, -3, and -7 which favour an aspartate residue in the P4 position and cleave targets with DEVD sequence. The third group contains caspases-6, -8, and -9 which exhibit certain degrees of flexibility in their P4 preferences with optimal substrate cleavage sequence (L/V)EXD and IEXD. These

Table 1. Members of the caspase family of proteases

Name	Alternative name	References
Caspase-1	ICE	Yuan et al., 1993b
Caspase-2	ICH-1, Nedd2	Wang et al., 1994; Kumar et al., 1994
Caspase-3	CPP32, Yama, apopain	Fernandes-Alnemri et al., 1994; Tewari et al., 1995b; Nicholson et al., 1995
Caspase-4	ICE_{rel}II, TX, ICH-2	Munday et al., 1995; Faucheu et al., 1995; Kamens et al., 1995
Caspase-5	ICE_{rel}III, TY	Munday et al., 1995; Faucheu et al., 1996
Caspase-6	Mch2	Fernandes-Alnemri et al., 1995a
Caspase-7	Mch3, ICE-LAP3, CMH-1	Fernandes-Alnemri et al., 1995b; Duan et al., 1996a; Lippke et al., 1996
Caspase-8	MACH, FLICE, Mch5	Boldin et al., 1996; Muzio et al., 1996; Srinivasula et al., 1996a
Caspase-9	ICE-LAP6, Mch6	Duan et al., 1996b; Srinivasula et al., 1996b
Caspase-10	Mch4, FLICE2	Fernandes-Alnemri et al., 1996; Vincenz et al., 1997
Caspase-11	ICH-3	S.Wang et al., 1996
Caspase-12		Van de Craen et al., 1997
Caspase-13	ERICE	Humke et al., 1998

results are consistent with the cleavage sites of the intracellular proteins known to be cleaved by caspases. For instance, caspase-1 cleaves pro-interleukin-1β at two sites with sequences of FEAD and YVHD (Cerretti et al., 1992; Thornberry et al.,1992). Caspase-3 cleaves a number of proteins at DXXD sequence during the induction of apoptosis, such as PARP containing DEVD motif (Lazebnik et al., 1994; Nicholson et al., 1995), whereas caspase-6 cleaves the nuclear envelope protein lamin A at VEID sequence (Takahashi et al., 1996). It is important to note, however, that these observations should not be interpreted such that one is somewhere near to establish the specific protein substrates of the individual caspases in cells undergoing apoptosis. In fact, genetic analysis has thus far failed to identify specific substrates of the caspases (reviewed by Villa et al., 1997). This may be probably due to the fact that the multiple caspases exist in cells and many of them have overlapping cleavage activities as demonstrated by a number of *in vitro* studies. To complicate this further, some intracellular protein substrates may also be

cleaved by non-caspase proteases during the induction of apoptosis. For example, actin, a major component of the cytoskeleton, may be cleaved by caspases (Mashima et al., 1995; Kayalar et al., 1996) as well as by non-caspase (Brown et. al., 1997) in cells undergoing apoptosis.

1.5.1.2 Role of caspases in apoptosis

Several lines of evidence demonstrate that caspases are critically involved in apoptosis. First, early studies have shown that over-expression of caspases-1 (Miura et al., 1993), -2 (Kumar et al., 1994; Wang et al., 1994), or -3 (Fernandez-Alnemri et al., 1994) induces apoptosis in various cell systems. Second, several procaspases are proteolytically activated early during apoptosis induced by a variety of apoptotic stimuli (Armstrong et al., 1996; Duan et al., 1996a; Orth et al., 1996; Shi et al., 1996; Faleiro et al., 1997; H. Li et al., 1997; MacFarlane et al., 1997; Medema et al., 1998). Thirdly, as mentioned above, many of the proteins being cleaved during apoptosis are cleaved at aspartate residues by caspases. Furthermore, specific inhibitors of caspases such as synthetic peptides, viral proteins, dominant-negative caspases, and antisense constructs can block apoptosis both *in vitro* and *in vivo* induced by a diverse proapoptotic stimuli (reviewed by Cohen, 1997; Villa et al., 1997; Cryns and Yuan, 1998). The most definitive support for their role in apoptosis comes from the *in vivo* studies with targeted gene knock-out approaches. Mice deficient in caspase-3 show a phenotype of decreased apoptosis in the brain and premature lethality (Kuida et al., 1996). Recently, it has been shown that mice deficient in caspase-9 die perinatally with a markedly enlarged and malformed cerebrum most probably caused by reduced apoptosis during brain development (Kuida et al., 1998; Hakem et al., 1998). Taken together, these findings suggest unequivocally that caspases are essential mediators of apoptosis, although not all caspases play an equally important role in apoptosis. For instance, evidence is accumulating that caspase-1 is of greater significance in inflammatory response than in apoptosis (reviewed by Chinnaiyan and Dixit, 1996; Cohen, 1997; Thornberry and Lazebnik, 1998), despite the fact that it is the first identified member of the caspase family of proteases.

How do caspases function in contributing to apoptotic cell death? Although the precise relationship of most cellular protein substrates cleaved by caspases to apoptosis is largely unknown, careful evaluation of the functions of these substrates may provide some clues. From a growing list of substrates that are cleaved by caspases during apoptosis (see Table

2), at least four classes of substrates with apparently different functions have emerged (reviewed by Villa et al., 1997; Thornberry and Lazebnik, 1998). Class 1 substrates include those enzymes whose activation requires caspase cleavage. Typical examples are the procaspases themselves, as described earlier. Class 2 substrates are structural proteins, as disassembly of cellular structure is an essential event leading to the demise of the cell. For example, cleavage of nuclear scaffold protein lamins is often observed in cells undergoing apoptosis, which probably contributes to the collapse of the nucleus and condensation of chromatin, classical features of apoptosis (Lazebnik et al., 1995; Takahashi et al., 1996; Rao et al., 1996). Class 3 substrates are those proteins whose function is to prevent cells from undergoing apoptosis. An example is the cleavage of DFF45/ICAD (Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998), an inhibitor of the nuclease responsible for DNA fragmentation known as CAD (caspase-activated deoxyribonuclease). Other inhibitors of apoptosis cleaved by caspases include Bcl-2 protein (Cheng et al., 1997). Class 4 substrates represent, however, the majority of the proteins whose consequence for apoptosis after caspase cleavage is not fully understood yet. These include PARP, DNA-PK, U1-70kDa and many more. It is speculated that the proteolytic disruption of critical homeostatic and repair functions of these proteins may facilitate cellular disassembly. Therefore, taken together, these findings clearly demonstrate that caspase-mediated proteolysis of many specific intracellular proteins is closely associated with the development of many characteristic features of apoptosis. Obviously further studies are required to elucidate the functional relationship between those known as well as newly discovered caspase substrates and the apoptotic phenotype.

Table 2. Proteins known to be cleaved by caspases during apoptosis

Proteins	Cleavage motif	References
Class 1: enzymes whose activation requires caspase cleavage		
procaspases	Y/VEX*D, DXXD,	Nicholson and Thornberry, 1997; Cohen,1997
pro-IL-1β	YVHD	Thornberry et al., 1992
PKC δ	DMQD	Emoto et al., 1995
MEKK-1	DXXD	Cardone et al., 1997
PAK2	?	Rudel and Bokoch, 1997
Class 2: components of structural framework		
nuclear lamins	VEID	Lazebnik et al., 1995; Takahashi et al., 1996
Gas2	SRVD	Brancolini et al., 1995
gelsolin		Kothakota et al., 1997
Class 3: inhibitors of apoptotic process		
DFF45/ICAD	DETD, DAVD	Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998
Bcl-2	DAGD	Cheng et al., 1997
Bcl-x_L	?	Clem et al., 1998
Class 4: substrates with unknown consequence for apoptosis		
PARP (DNA repair)**	DEVD	Lazebnik et al., 1994; Nicholson et al., 1995
DNA-PK_{CS} (DNA repair)	DEVD	Casciola-Rosen et al., 1995; Song et al., 1996
U1-70 kDa (component of mRNA splicing complex)	DGPD	Casciola-Rosen et al., 1994; Tewari et al., 1995a
SREBPs (sterol regulatory element binding protein)	DEPD	X.Wang et al., 1996
heteroribonuclear proteins C1 and C2 (mRNA processing)	?	Waterhouse et al., 1996
DSEB/RF-C140 (subunit of DNA replication complex c)	DEVD	Ubeda and Habener, 1997
D4-GDI (regulating Rho GTPases)	DELD	Na et al., 1996
fodrin (membrane associated cytoskeletal protein)	DETD	Martin et al., 1995a; Cryns et al., 1996; Vanags et al., 1996
actin (cytoskeletal protein)	?	Mashima et al., 1995; Kayalar et al., 1996
Rb (cell cycle regulatory protein)	DEAD	Janicke et al., 1996; Tan et al., 1997
Huntintin (huntinton disease gene product)	DXXD	Goldberg et al., 1996
β-catenin (cell-cell contact)	?	Brancolini et al., 1997
presenilins 1 and 2	?	T.Kim et al., 1997; Vito et al., 1997

* denotes any amino acid residue

** known functions indicated in bracket

1.5.1.3 Mechanisms of caspase activation

As described earlier, caspases are present in cells as inactive proenzymes which require activation. Once activated by proteolytic processing at aspartate residues, they are capable of activating other caspases as well as cleaving intracellular protein substrates. But how is the first caspase activated? The available evidence suggests that there exists at least two distinct initial caspase activating mechanisms, as outlined in figure 2.

The first of these pathways is initiated in response to apoptotic stimuli such as DNA damaging agents that trigger a release of mitochondrial cytochrome c into the cytoplasm, where it interacts with other molecules to form a caspase cascade initiating complex. This finding was based on an early observation that cytochrome c, a key component of the mitochondrial electron transport chain, is required for the activation of caspase-3 in a cell-free system (Liu et al., 1996). Further studies show that cytochrome c is rapidly released from the mitochondria into the cytoplasm during the induction of apoptosis by diverse apoptotic stimuli in several cell systems (Krippner et al., 1996; Yang et al., 1997; Kluck et al., 1997). Significantly, cytochrome c release occurs prior to caspase-3 activation and DNA fragmentation in cells undergoing apoptosis induced by staurosporine, UV irradiation or etoposide (Yang et al., 1997; Kluck et al., 1997; Bossy-Wetzel et al., 1998). Cytosolic extracts that have been removed of cytochrome c by immunodepletion are unable to activate caspase-3 or induce DNA fragmentation, and re-addition of holocytochrome c (containing heme) but not apocytochrome c (without heme) restores these activities (Liu et al., 1996). Meanwhile, over-expression of anti-apoptotic Bcl-2 or Bcl-xL protein prevents apoptosis as well as translocation of cytochrome c from mitochondria to the cytoplasm in cells treated with apoptotic stimuli including DNA damaging agents and staurosporine (Yang et al., 1997; Kluck et al., 1997; Vander Heiden et al., 1997; Kharbanda et al., 1997). These results clearly indicate that cytochrome c release is an early event during the induction of apoptosis, and yet Bcl-2 or Bcl-xL is capable of blocking its release thus acting upstream of this event. Recent studies demonstrate that, upon its release into the cytoplasm, cytochrome c, together with dATP, Apaf-1 (mammalian Ced-4 homologue) and procaspase-9, forms a complex, which results in the activation of caspase-9 (P. Li et al., 1997; Zou et al., 1997). Sequence and structural analyses show that Apaf-1 contains three functional domains, an amino-terminal caspase-recruitment domain (CARD), a conserved nucleotide binding domain in the middle and a carboxy-terminal domain with many WD repeats presumably important

for protein-protein interaction (Zou et al., 1997). Binding of Apaf-1 to caspase-9 through their respective amino-terminal CARD domains requires both dATP and cytochrome c, suggesting that dATP and cytochrome c may function to alter the conformation of Apaf-1, rendering its CARD domain more accessible to caspase-9 (P. Li et al., 1997). dATP interacts with the nucleotide domain in Apaf-1 that is conserved in Ced-4, where mutations of this region in Ced-4 impair its ability to activate Ced-3 (Chinnaiyan et al. 1997a; Seshagari and Miller, 1997). Once bound to Apaf-1, caspase-9 undergoes autoproteolytic activation which subsequently activates other caspases such as caspase-3 (P. Li et al., 1997; Srinivasula et al., 1998). Consistent with the role of caspase-9 as an apical caspase in this model of caspase activation cascade, over-expression of a catalytically inactive caspase-9 mutant fails to activate caspase-3 and attenuate the apoptotic response (P. Li et al., 1997). Recently, Bcl-xL has been shown to bind specifically to the Ced-4-like domain of Apaf-1 and inhibit Apaf-1-dependent caspase-9 activation (Hu et al., 1998), whereas pro-apoptotic members of Bcl-2 family proteins Bax and Bak can disrupt interaction between Bcl-xL and Apaf-1 (Pan et al., 1998). These observations provide further evidence for a role of cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex in initiating the caspase activation cascade.

The second pathway of initiating caspase activation starts from the cell surface membrane with ligand binding to a death receptor. Apoptosis mediated by ligation of death receptor CD95, for example, involves the initial formation of a death inducing signalling complex (DISC), which is formed following the recruitment of an adaptor molecule, FADD (Fas-associating protein with a death domain) (Kischkel et al., 1995). FADD binds through its death effector domain (DED) to one of the two DEDs in the prodomain of caspase-8, resulting in its activation (Boldin et al., 1996; Muzio et al., 1996). Once activated, caspase-8 then activates other caspases resulting in the apoptotic phenotype. This pathway thus provides a direct link between cell death receptors and the caspases. Recently, it has been shown that, apart from direct activation of other caspases, active caspase-8 can also cleave other cytosolic factor(s), such as Bid (Bcl-2 interacting protein with proapoptotic BH3 domain), resulting in a translocation of the cleaved Bid to mitochondria where it induces the release of cytochrome c (Li et al., 1998; Luo et al., 1998). This results in activation of caspase-9, which in turn activates other caspases (Srinivasula et.al., 1996a; Scaffidi et al., 1998). Thus, depending on the cell type, mitochondria may be initially bypassed in death receptor-mediated apoptosis following

direct activation of the downstream caspases such as caspases-3 and -7 by caspase-8 (Scaffidi et al., 1998). The downstream caspases then cleave cellular protein substrates as well as disrupt mitochondrial membrane function leading to a release of mitochondrial cytochrome c, resulting in a further activation of caspases and the establishment of a self-amplification loop (Marzo et al., 1998; Kuwana et al., 1998), so accelerating the apoptotic process.

Interestingly, in both pathways the importance of the prodomains of caspases in the regulation of their activation has been highlighted by the fact that the prodomains mediate protein-protein interactions. The CARD and DED domains in the prodomains of caspases-9 and -8, respectively, all function as binding sites between two molecules leading to the initiation of caspase activation. These caspases with long prodomains are thus also known as initiation caspases, whereas other caspases with no or short prodomains such as caspase-3, -6, and -7 are referred to as effector caspases (Cohen, 1997). Therefore the execution of the death programme appears to occur following activation of a hierarchy of caspases with caspase-8 and caspase-9 being at the apex of the cascades originating at the cell membrane or the mitochondria respectively (Figure 2).

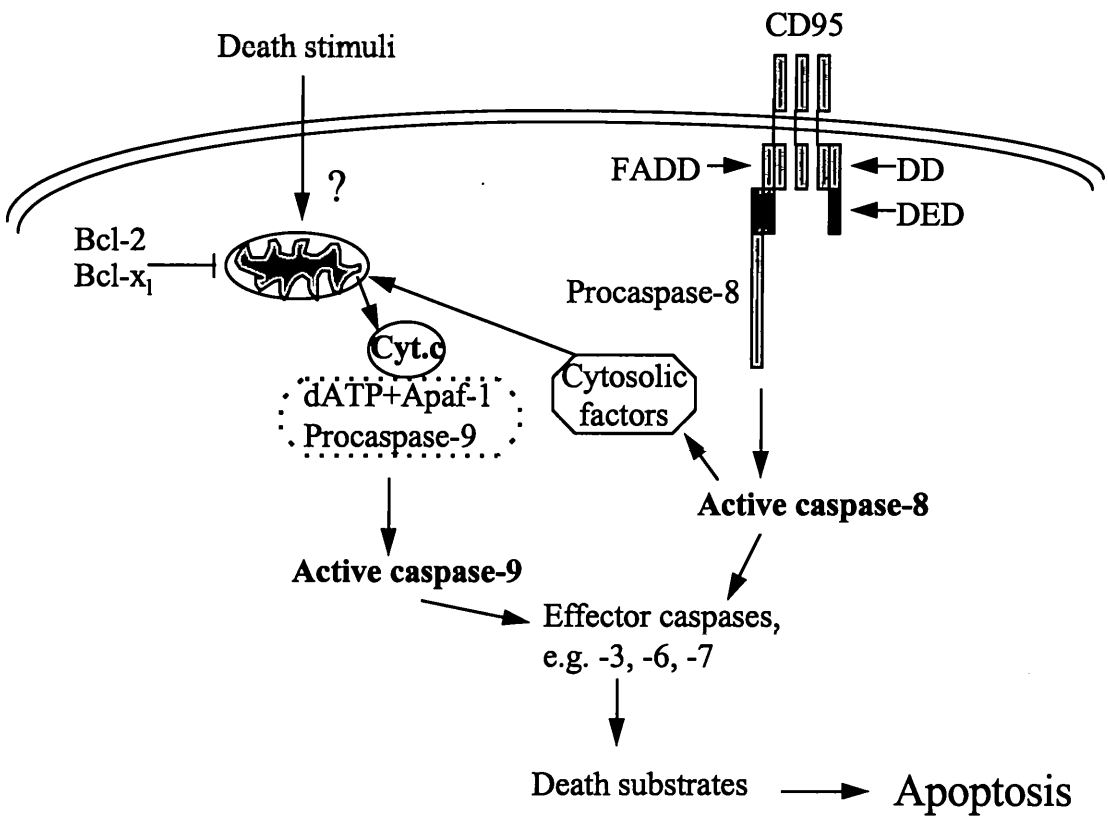


Figure 2. Two distinct pathways that initiate caspase activation cascade

1.5.1.4 Inhibitors of caspases

The importance of inhibitors in the regulation of complex proteolytic systems in general is well recognised, and this is no exception in the control of cell death specific proteases. In fact, it has been suggested that the inhibitors can establish a threshold that regulates the concentration of active caspases in the cell, thus preventing the undesirable cell death resulting from spontaneous activation (Thornberry and Lazebnik, 1998). These inhibitors can be subdivided into two groups based on their origins.

As described in section 1.3.1.3, identification of caspase inhibitors has come from the work on viruses, which inhibit apoptosis to circumvent the normal host defence to infection. These inhibitors include cowpox protein CrmA (Ray et al., 1992), baculovirus proteins p35 (Clem et al., 1991; Bump et al., 1995) and a family of IAP (inhibitor of apoptosis) (Clem and Miller, 1994; Hawkins et al., 1996). CrmA is a serpin-like protease inhibitor and over-expression of CrmA prevents apoptosis in a number of cell systems (Miura et al., 1993; Gagliardini et al., 1994). Although CrmA was originally described as a specific inhibitor of caspases (Ray et al., 1992), it is now known that it inhibits different caspases in a differential manner. It is a potent inhibitor of caspases-1, -4, -6 and -8, but less effective in inhibiting caspases-2, -3, -7, and -10 (reviewed by Cohen, 1997), which suggests that there is CrmA-sensitive and -resistant pathways present in the cells. Consistent with this, CrmA has been shown to block apoptosis induced by CD95, TNFα, and growth factor withdrawal, but not cell death induced by DNA damaging agents, staurosporine or over-expression of pro-apoptotic member of Bcl-2 family protein Bak (reviewed by Cohen, 1997; Villa et al., 1997). The baculovirus p35 protein exhibits apoptosis-protective effect in a manner similar to CrmA, but with broader spectrum in inhibiting caspases. p35 has been shown to inhibit caspases-1, -2, -3 and -4 *in vitro* and block apoptosis induced by CD95, TNFα, and growth factor withdrawal as well as DNA damaging agents (Villa et al., 1997). However, although there is no reported instances of p35-resistant apoptosis, its selectivity for caspases is not clearly defined yet. The precise caspase targets of a newly discovered family of IAP proteins also remain to be elucidated. IAPs inhibits caspases-3 and -7 *in vitro*, suggesting that they block apoptosis by inhibiting caspases (Deveraux et al., 1997). In contrast to p35 and CrmA that require peptide bond hydrolysis as part of their inhibitory mechanism (Komiyama et al., 1994; Xue and Horvitz, 1995), IAPs act as inhibitors not as substrates of the caspases (Roy et al., 1997). Future studies obviously need to address the mechanism by which IAPs inhibit caspases.

Synthetic inhibitors are mainly the peptides which contain a substrate recognition motif similar to that found in the endogenous substrates, such as YVAD and DEVD, in order to be selective for caspases. There are reversible inhibitors comprising mostly the aldehyde derivatives as well as irreversible inhibitors containing chloromethyl, fluoromethyl and acyloxymethyl ketone groups (reviewed by Thornberry, 1994; Cohen, 1997; Villa et al., 1997). However, the specificity of the peptide inhibitors is always subject to dispute. In addition, their use is also limited by membrane impermeability in intact cells (reviewed by Henkart, 1996). To overcome the pitfall, a tripeptide inhibitor, benzyloxycarbonyl-valinyl-alanyl-aspartyl (OMe) fluoromethyl ketone (Z-VAD.FMK) has been developed. It, at lower micromolar concentration, inhibits the processing of caspase-3 *in vitro* (Slee et al., 1996) and DEVD cleavage activity in lysates from cells pre-treated with apopotic stimuli (MacFarlane et al., 1997). This tripeptide is a cell-permeable inhibitor of caspases whose permeability is facilitated by the presence of benzyloxycarbonyl and OMe groups (Cohen, 1997). It inhibited apoptosis induced by a variety of stimuli in a number of cell systems, including rat thymocytes (Fearnhead et al., 1995a) and hepatocytes (Cain et al., 1996), human leukaemic Jurkat T cells (Chow et al., 1995; Armstrong et al., 1996), human B-cell lymphoma cell line GM701 and fibroblast cell line SKW6.4 (Jacobson et al., 1996) and human monocytic THP.1 cells (Zhu et al., 1995). Z-VAD.FMK also blocks CD95-induced apoptosis in the liver of mice (Rodriguez et al., 1996; Chandler et al., 1998). Thus careful use of synthetic peptide inhibitor is helpful to elucidate the role of caspases, particularly in intact cell systems.

1.5.2 Mitochondrial changes

When apoptosis was first described as a unique type of cell death distinct from necrosis, the ultrastructure of cytosolic organelles, including mitochondria, appeared to be well preserved and the cytoplasm became condensed as a result of overall shrinkage in cells undergoing apoptosis (Kerr, 1971; Kerr et al., 1972). Subsequent studies consistently show a nearly normal appearance of mitochondria (reviewed by Wyllie et al., 1980; Arends and Wyllie, 1991). These morphological findings are further confirmed by a recent study investigating changes in mitochondrial function during apoptosis (Krippner et al., 1996). The preservation of mitochondrial structure and function is also consistent with the observations that a normal supply of ATP provided by intact mitochondria is required for an active and controlled apoptotic process to proceed (Leist et al., 1997;

Eguchi et al., 1997; Stefanelli et al., 1997). However, since the recognition that the anti-apoptotic Bcl-2 protein is localised on the mitochondrial membrane (Hockenbery et al., 1990; Krajewski et al., 1993), many attempts have been made to ascertain a role of mitochondria in apoptosis in addition to its primary role in energy production.

1.5.2.1 Bcl-2 family proteins and mitochondria

At least 15 Bcl-2 family proteins have so far been identified in mammalian cells, which consist of both cell death promoters and inhibitors, such as the anti-apoptotic proteins Bcl-2, Bcl-x_L, Mcl-1 and the pro-apoptotic proteins Bax, Bcl-x_S, Bak, Bid (reviewed by Nunez and Clarke, 1994; Reed, 1997a; Chao and Korsmeyer, 1998; Adams and Cory, 1998). Many of these proteins reside in the outer mitochondrial membrane, anchored by a stretch of hydrophobic amino acids located at the carboxyl terminus with the proteins oriented towards the cytosol (reviewed by Reed et al., 1998). Thus the obvious assumption is that the association of Bcl-2 family proteins with mitochondria may be required for their appropriate functions in regulating apoptosis. An early attractive hypothesis was that during apoptosis reactive oxygen species (ROS) generated from the mitochondrial electron transport chain could provide an apoptotic signal and Bcl-2 could function as an antioxidant. This was indeed supported by the observation that over-expression of Bcl-2 inhibited both the nuclear changes of apoptosis and mitochondrial generation of ROS (Hockenbery et al., 1993; Kane et al., 1993). However, cells without the functional electron transport chain due to the lack of mitochondrial DNA could still be induced to undergo apoptosis (Jacobson et al., 1993; Marchetti et al., 1996a). Furthermore, when cultured under anaerobic conditions where the generation of ROS is limited, cells are able to undergo apoptosis in hypoxia condition alone (Shimizu et al., 1995) or induced by either ligation of death receptor CD95 or withdrawal of survival factor (Jacobson and Raff, 1995), which are all inhibited by Bcl-2. Thus, the oxidant signalling hypothesis does not appear to explain a general role of mitochondria in apoptosis and Bcl-2 can protect cells against apoptosis by a mechanism independent of inhibiting the production and/or effects of ROS.

Currently, there are two major mitochondrial events associated with apoptosis: 1) release of apoptogenic proteins from mitochondria into the cytosol; and 2) opening of the mitochondrial permeability transition (PT) pore, which results in a reduction in the mitochondrial membrane potential ($\Delta\psi_m$). The Bcl-2 family proteins have, with no

exception, been implicated in the regulation of these two important events, as described below.

1.5.2.2 Release of apoptogenic proteins from mitochondria

Two proteins have been reported to be capable of inducing nuclear apoptotic changes when released from mitochondria into the cytosol, i.e. cytochrome c and apoptosis inducing factor (AIF) (Liu et al., 1996; Susin et al., 1996a). As described earlier in section 1.5.1.3, cytochrome c, together with dATP, Apaf-1 and procaspase-9, forms a complex, which results in the activation of caspase-9 (P. Li et al., 1997; Zou et al., 1997). Over-expression of anti-apoptotic Bcl-2 and Bcl-x_L proteins prevents translocation of cytochrome c from mitochondria to cytoplasm in cells treated with diverse apoptotic stimuli (Yang et al., 1997; Kluck et al., 1997; Vander Heiden et al., 1997; Kharbanda et al., 1997). Conversely, addition of recombinant Bax protein to isolated mitochondria induces cytochrome c release (Jurgensmeier et al., 1998). Although the mechanisms by which Bcl-2 prevents and Bax promotes the release of cytochrome c from mitochondria are not clear, these observations provide supportive evidence of a link between the regulation of apoptosis by Bcl-2 family proteins and mitochondria.

AIF alone, on the other hand, has been shown to directly activate caspases *in vitro*, resulting in proteolytic processing of procaspases and formation of active caspases (Susin et al., 1996a). It has, however, been argued that AIF itself is possibly a caspase-like protease based on the observation that purified AIF from the supernatants of depolarised mitochondria is able to cleave recombinant procaspase-3 through a mechanism that is inhibited by a tripeptide caspase inhibitor Z-VAD.FMK (Susin et al., 1997). Over-expression of anti-apoptotic Bcl-2 protein prevents the release of AIF from mitochondria to the cytoplasm in cells treated with several different apoptotic stimuli (Susin et al., 1996a and 1997), providing further evidence that Bcl-2 family proteins can regulate apoptosis at mitochondria. It, however, has to be noted that, to date, cDNAs encoding AIF have not been cloned and thus the molecular identity of the protein still remains to be determined.

1.5.2.3 Reduction in mitochondrial membrane potential ($\Delta\psi_m$)

During oxidative phosphorylation, the synthesis of ATP is tightly coupled to a flow of electrons from NADH or $FADH_2$ to O_2 by a proton gradient across the inner

mitochondrial membrane (Nicholls and Ferguson, 1992). Electron flow through three asymmetrically oriented transmembrane complexes (also known as respiratory chains) results in the pumping of protons out of the mitochondrial matrix into the intermembrane space. As a result, a proton-motive force is generated, which consists of a pH gradient (cytosolic side acidic) and a membrane potential (cytosolic side positive). The energy stored in either the pH gradient or the membrane potential drives the F_0F_1ATPase (also known as ATP synthase) in the inner mitochondrial membrane to synthesise ATP when the protons re-enter the mitochondrial matrix. Under physiological situations, mammalian mitochondria express this electrochemical gradient mostly as membrane potential at about 180 mV and to a less extent as pH gradient equivalent to 60 mV, which together yield a total energy of 240 mV (reviewed by Chen, 1988). Thus, the membrane potential is essential for mitochondrial production of ATP. The mitochondrial membrane potential is also believed to play a vital role in the import of precursor proteins (reviewed by Geli and Glick, 1990; Bauer et al., 1996), intracellular Ca^{++} homeostasis (reviewed by Gunter and Gunter, 1994; Budd and Nicholls, 1996a) and mitochondrial protein synthesis (Abou-Khalil et al., 1986).

Reduction in the mitochondrial membrane potential ($\Delta\psi_m$) has recently been observed to occur early during the induction of apoptosis by a variety of stimuli (Petit et al., 1995; Zamzami et al., 1995) and to possibly regulate the aberrant exposure of phosphatidylserine on the outer plasma membrane of the cells undergoing apoptosis (Castedo et al., 1996). The decrease in $\Delta\psi_m$ occurs as a result of the opening of the permeability transition (PT) pore or megachannel in the inner mitochondrial membrane (reviewed by Petit et al., 1996; Susin et al., 1996b). Although the PT pore has been known for more than 20 years (Hunter et al, 1976), to date the structure and composition of the PT pore still remain poorly defined and so does its physiological function. It appears to be a complex of polypeptides located presumably at inner and outer membrane contact sites and is possibly composed of both inner membrane proteins, such as the adenine nucleotide translocator (ANT) and cyclophilin D, and outer membrane proteins, such as porin (also known as voltage dependent anion channel) which operate in concert to create a channel (reviewed by Bernardi et al., 1994). The PT pore thus probably represents the channel in which mitochondria operate mainly to release Ca^{++} and participate in the intracellular Ca^{++} signalling network necessary for many physiological functions of the cell and its opening also allows a permeability of the inner mitochondrial

membrane to solutes of molecular mass less than about 1.5 kDa (reviewed by Zoratti and Szabo, 1995). Ca^{++}, P_i, and oxidant chemicals induce the opening of PT pore, whereas Mg^{++}, ADP, low pH and high membrane potential oppose the occurrence of PT. The rapid change in permeability resulting from the opening of PT pore causes loss of membrane potential, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, and mitochondrial swelling (Zoratti and Szabo, 1995). It has also been shown that the induction of PT in isolated mitochondria results in the release of AIF capable of causing isolated nuclei to undergo chromatin condensation and DNA fragmentation (Marchetti et al., 1996b). Inhibition of PT by pharmacological agents or by over-expression of Bcl-2 prevented apoptosis (Susin et al., 1996a and 1997), whereas over-expression of the pro-apoptotic protein Bax induces a loss of $\Delta\psi_m$ and apoptosis (Xiang et al., 1996). Thus it has been proposed that a reduction in mitochondrial membrane potential ($\Delta\psi_m$) is a critical event of apoptosis (Kroemer et al., 1997).

The observations that over-expression of Bcl-2 and Bcl-x_L or Bax proteins prevents or promotes the release of apoptogenic proteins from mitochondria and opening of the mitochondrial permeability transition (PT) pore during the induction of apoptosis, as described above, provide an important insight into the mechanisms whereby Bcl-2 family proteins regulate apoptosis in mitochondria. Recent studies have shown, however, that Bcl-2 and Bcl-x_L also protect against apoptosis in cells microinjected with cytochrome c (F. Li et al., 1997; Zhivotovsky et al., 1998). This suggests that Bcl-2 family proteins may also utilise other mechanism(s) of action apart from the mitochondrial ones. Evidence is now accumulating that some Bcl-2 family proteins are multifunctional, possessing more than one mechanisms for modulating cell death. These include their functions in channel formation and ion movement, action as a chaperone or molecular gatekeeper to control movement of proteins such as cytochrome c across the membranes, or as an adapter or docking protein to either facilitate or block interaction of proteins leading to activation of caspases, and as a protein that preserves integrity of membranes (reviewed by Reed, 1997a; Cai et al., 1998; Chao and Korsmeyer, 1998; Reed et al., 1998).

1.5.3 Cell surface changes leading to recognition and phagocytosis

One of the key features associated with apoptosis *in vivo* is the rapid phagocytosis of cells undergoing apoptosis by phagocytes (Kerr et al., 1972; Wyllie et al., 1980). This protects surrounding tissues from exposure to injurious contents leaking from dying cells, thereby preventing the occurrence of inflammation. The speed of phagocytic clearance also suggests that phagocytosis is an efficient means of eliminating apoptotic cells. This swift phagocytosis can be mediated by macrophages as well as neighbouring epithelial cells (reviewed by Savill et al., 1993). Currently, there is, however, only a limited understanding of the molecular mechanisms which render apoptotic cells recognisable to phagocytes (Savill, 1998). Nevertheless, it is apparent that the surface of cells undergoing apoptosis must be different from that of normal cells so that only the dying cells are labelled for disposal. In addition, the available data suggest that the cell surface signals leading to recognition and phagocytosis are derived from modification of existing plasma membrane components of the cell, as will be described below.

1.5.3.1 Carbohydrates changes

One early observation showed that rodent thymocytes undergoing apoptosis induced by glucocorticoids exhibits a reduced mobility in a cell micro-electrophoresis assay consistent with loss of sialic acids (Morris et al., 1984). Further study showed that mono- and disaccharides inhibit recognition of apoptotic thymocytes by macrophages (Duvall et al., 1985), suggesting that loss of terminal sialic acid residues from the sugar side chains of cell surface glycoproteins of the apoptotic cells may expose normally masked residues such as N-acetyl glucosamine, N-acetyl galactosamine and galactose, which are recognised by macrophage lectins, one putative mechanism of cellular interaction (Sharon and Lis, 1989). These results also imply that, as a consequence of apoptosis, cells undergo specific changes in surface carbohydrates. Further support comes from the observation that in neonatal rat liver cell cultures the asialoglycoprotein receptor has been implicated in the phagocytosis of apoptotic cells by healthy hepatocytes (Dini et al., 1992). However, specific exposure of such sugars by apoptotic cells has not been consistently demonstrated (Morris et al., 1984) and further studies are needed to clarify the sites and timing of carbohydrate changes occurring on the surface of apoptotic cells.

1.5.3.2 Thrombospondin binding moiety

Anionic thrombospondin binding sites on the surface of apoptotic cells also appear to be important in signalling recognition of apoptotic cells by a number of phagocyte types.

This is based on the observation that recognition of apoptotic neutrophils by human monocyte-derived macrophages is specifically inhibited by aminosugars and amino acids in a charge-dependent manner (Savill et al., 1989). Varying the pH at which the interaction was performed not only demonstrates a correlation of phagocytosis with pH, but also indicates that the inhibitory effects of these small molecules depended on carrying a cationic charge. Furthermore, pre-incubation experiments show that aminosugars exert their effects at the surface of apoptotic neutrophils, suggesting the involvement of particular anionic groups on the neutrophil surface (Savill et al., 1989). The inhibitory effects of cationic aminosugars also suggest that these anionic sites might bind thrombospondin, a trimeric multifunctional adhesive glycoprotein secreted by many cell types (Frazier, 1987). Subsequent studies have indeed shown that apoptotic neutrophils exhibit an increased binding to latex beads coated with thrombospondin, which could be inhibited by the mouse monoclonal antibodies that also inhibits recognition of apoptotic cells by monocyte-derived macrophages (Savill et al., 1992). Thus, these observations indicate that thrombospondin acts as a bridge between the apoptotic neutrophils and macrophages. Meanwhile, the phagocyte adhesion receptor CD36 is found to co-operate with the vitronectin receptor integrins ($\alpha_v\beta_3$) to bind thrombospondin (Savill et al., 1990; Fadok et al., 1992a). However, it must be pointed out that these findings are all obtained from cultured neutrophils undergoing apoptosis and thus the significance of the findings needs to be verified in other cell systems. In addition, the anionic thrombospondin binding sites on the surface of apoptotic cells remain to be further characterised.

1.5.3.3 Externalisation of phosphatidylserine

Phosphatidylserine (PS), an anionic phospholipid normally confined to the inner leaflet of the plasma membrane bilayer by an ATP-dependent aminophospholipid translocase (Verhoven et al., 1995; Tang et al., 1996), is currently the best characterised signal for recognition. This mechanism was initially suggested by the observation that macrophages bind resealed red cell ghosts that apparently lost asymmetry of membrane phospholipids (McEvoy et al., 1986). Normally, the distribution of membrane phospholipids is asymmetric, with the outer leaflet of the membrane bilayer predominantly containing the neutral phospholipids sphingomyelin and phosphatidylcholine, while anionic phospholipids such as phosphatidylserine being restricted to the inner leaflet (Op den Kamp, 1979). Later, exposure of PS to the external leaflet of the plasma membrane

bilayer is observed as a general phenomenon associated with apoptosis regardless of the initiating stimulus (Fadok et al., 1992b). Further studies have shown that PS exposure is an early feature of apoptosis in a number of systems, serving as a recognition signal for yet to be characterised PS receptors on certain phagocyte populations (Fadok et al., 1992b; Martin et al., 1995b). The mechanisms mediating PS exposure are complex but seem to involve both downregulation of the amino phospholipid translocase activity and activation of a non-specific lipid scramblase (Verhoven et al., 1995; Bratton et al., 1997). Recently, both activation of caspases and loss of mitochondrial membrane potential have also been implicated in PS exposure in cells undergoing apoptosis (Vanags et al., 1996; Martin et al., 1996; Castedo et al., 1996; Zamzami et al., 1996). However, the precise mechanisms of PS exposure still remain elusive.

In summary, one of the key features of apoptosis is that cells undergoing this form of cell death are swiftly recognised by phagocytes and ingested while still intact, protecting tissues from the potentially harmful consequences of exposure to the contents of the dying cells. Therefore a critical event of apoptosis involves the acquisition of plasma membrane changes that result in the recognition and engulfment of dying cells by phagocytes. To date, the nature and kinetics of these surface changes, however, remain largely unknown. Different membrane changes may lead to recognition and removal of apoptotic cells by different types of phagocytes. Similarly, phagocytes may utilise multiple systems for recognition of apoptotic cells, as recently indicated (Pradhan et al., 1997). Other signals for recognition may also be involved, which may play an important role in phagocytosis of the apoptotic cells. Further studies are thus required for elucidating the mechanisms of bringing about the signals for recognition as well as revealing their significance in clearance of apoptotic cells *in vivo*.

1.6 Framework of the study

1.6.1 Rationale

Apoptosis, a basic form of cell death which plays a major role in the development and homeostasis of multicellular organisms, is also an important form of cell death induced by anti-cancer drugs and toxic chemicals (reviewed by Hickman, 1992; Corcoran et al., 1994; Trump et al., 1997). However, the biochemical mechanisms of chemical-induced apoptosis remain largely unknown. In addition, as reviewed above, apoptosis is a programmed form of cell death characterised by morphological and biochemical changes

affecting the nucleus, cytoplasm and plasma membrane. These changes in various cellular compartments are widely regarded as mechanistically linked events in a single "program", in which activation of caspases and proteolysis of intracellular substrates represent a final common pathway leading to cell death. To date there has, however, been very limited knowledge on the temporal relationship between these changes during apoptosis, particularly induced by chemicals.

1.6.2 Objectives

To facilitate the understanding of the biochemical mechanisms of apoptosis, I aimed to examine the importance of the major biochemical changes, particularly the activation of caspases, reduction in mitochondrial membrane potential and externalisation of PS in chemical-induced apoptosis. I also wished to study the temporal relationship of these biochemical events leading to the morphological and biochemical features of apoptosis. Finally, I intended to investigate the role of mitochondria in this model of apoptosis, in particular, its role in initial activation of caspases.

Chapter 2

Materials and methods

2.1 Materials

RPMI 1640 medium and foetal calf serum (FCS) were purchased from Life Technologies Ltd. (Paisley, Scotland). N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) was from Boehringer-Mannheim UK (Lewes, England). Benzyloxycarbonyl-valinyl-alanyl-aspartyl (OMe) fluoromethyl ketone (Z-VAD.fmk) was from Enzyme Systems Inc.(Dublin, CA, USA). "AnalaR" zinc acetate dihydrate was from BDH/Merck Ltd.(Poole, England). Etoposide, antimycin A, oligomycin (65 % oligomycin A), tumour necrosis factor-α (TNF-α) and all other chemicals were obtained from Sigma Chemical Company (Poole, England), unless otherwise stated.

2.2 Cell culture

2.2.1 Human monocytic THP.1 cells

THP.1 cells were obtained from European Collection of Animal Cell Cultures (ECACC) (Porton Down, England) and grown as a suspension in the medium supplemented with 10 % heat-inactivated FCS and 2 mM L-glutamine in an atmosphere of 5% CO_2 in air at 37°C. Cells were passaged on every third day and the cell density was maintained at 0.25-1 x 10^6/ml. Cell growth was assessed by counting cell numbers using a Scharfe cell counter with CASY II software. The population doubling time was between 32 and 36 hours. Logarithmically growing cells were used for all experiments with an average density of 0.5 x 10^6 cells/ ml.

2.2.2 Human leukaemic U937 cells

U937 cells were obtained from ECACC and maintained as a suspension under the same culture condition as THP.1 cells. Cells were kept in logarithmic growth by allowing each subculture to obtain a population density of about 1 x 10^6 cells/ml before re-seeding at 1 x 10^5 cells/ml. The population doubling time was between 20 and 25 hours as assessed by counting cell numbers using a Scharfe cell counter with CASY II software. Logarithmically growing cells were used for all experiments with an average density of 0.5 x 10^6 cells/ ml.

2.3 Microscopy

2.3.1 Fluorescence microscopy

The apoptotic morphological changes were initially examined under the fluorescent microscope with ultraviolet (UV) illumination (352 nm) following staining of the cells with the membrane permeable DNA binding dye, Hoechst 33342. Cells (1 x10^6 cells/ ml) were incubated with 30 µl of Hoechst 33342 (50 µg/ml) for 5 min at 37°C. After centrifugation at 200g for 3 min at 4 °C, cells were re-suspended in 300 µl of ice cold PBS containing 20 µl of propidium iodide (PI, 50 µg/ml). Cells taking up Hoechst 33342 were stained blue and considered viable, whereas those cells with PI inclusion were stained red and considered non-viable. Observed apoptotic changes included condensation and fragmentation of the nuclei.

2.3.2 Electron microscopy

The ultrastructural features of apoptosis were also examined under the electron microscope. Cells (2 x 10^6) were harvested as a pellet and processed as described (Cohen et al., 1992). Duplicate samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C overnight and postfixed with 1% osmium tetroxide/1% potassium ferrocyanide overnight at 4 °C. After fixation, cells were stained *en bloc* with 5% aqueous uranyl acetate overnight at room temperature, dehydrated, and embedded in Agar 100 epoxy resin. Sections, up to 1 µm were examined unstained by electron spectroscopic imaging using a Zeiss 902A electron microscope. Ultrathin sections were stained with lead citrate and examined in a Jeol 100-CXII electron microscope equipped with a rotating stage/eucentric goniometer.

2.4 Flow cytometry

2.4.1 Introduction

Flow cytometry is a technique for making rapid measurements on particles or cells as they flow in a fluid stream one by one through a sensing point. The feature of the technique is that it measures each event individually rather than as average values for the whole population. Typically, a flow cytometer consists of a fluidic system, detection devices, signal processing and display units (Ormerod, 1990). The fluidic system serves the purpose of delivering cells singly to a specific point in space intersected by, in most cases, a laser beam. This is achieved by forcing a cell suspension, usually with positive air pressure, into an enclosed channel through which sheath fluid is flowing. When cells travel as a stream of single particles, they are

intersected by a laser light. As a result, cells scatter and/or fluoresce depending on the method of staining. The scattered and fluorescent light generated by cells passing through the laser beam is collected by photodetectors which convert the photon pulses into electronic signals. Further electronic and computational processing results in a graphic display and quantitative analysis of measurements.

2.4.2 Hoechst 33342 staining

Hoechst 33342 is one of a family of bis-benzimidazoles which stains DNA specifically, binding preferentially to repetitive AT sequences in the minor groove of the helix. Unlike other DNA binding dyes which do not freely cross an intact plasma membrane, Hoechst 33342 is membrane permeable and also actively transported in and out of cells. It has been observed that cells undergoing apoptosis fluoresce more brightly than normal cells after a brief incubation with Hoechst 33342. Based on this observation, a flow cytometric method has been developed in the laboratory to separate and quantify viable apoptotic cells (Sun et al., 1992). Further study showed that the increased blue fluorescence is due to an increase in cell membrane permeability of apoptotic cells (Ormerod et al., 1993).

Briefly, 1 x10^6 cells were incubated with Hoechst 33342 (1.5 μg/ml) in RPMI 1640 medium containing 10 % FCS at 37 °C for 1 min with THP.1 or U937 cells. The cells were then cooled down on ice for 3 min, centrifuged at 200g for 3 min, re-suspended in 1 ml PBS containing 2.5 μg of propidium iodide (PI). Analysis was carried out at a flow rate of 200 cells/second on a Becton Dickinson Vantage flow cytometer with Lysis II software. Hoechst 33342 and PI were excited using the 352 nm UV line of a Krypton laser and resultant blue (420-480 nm) fluorescence with linear amplification versus red (~640 nm) fluorescence with logarithmic amplification were recorded. Non-viable, red fluorescent cells were gated out. The remaining cells were displayed as a cytogram of blue fluorescence against forward light scatter (indicative of cell size). Viable cells with high blue fluorescence was determined as apoptotic cells.

2.4.3 Detecting phosphatidylserine with binding of annexin V

One of the key features associated with apoptosis *in vivo* is the rapid phagocytosis of apoptotic cells by phagocytes (Arends and Wyllie, 1991). This protects surrounding

tissues from exposure to injurious contents leaking from dying cells, thereby preventing the occurrence of inflammation. Currently there is only a limited understanding of the molecular mechanisms which render apoptotic cells recognisable to phagocytes (reviewed by Savill et al., 1993). However, exposure of phosphatidylserine (PS) in the external leaflet of the plasma membrane lipid bilayer, a general phenomenon of apoptosis regardless of the initiating stimulus, appears to serve as a recognition signal for yet to be characterised PS receptors on certain phagocyte populations (Fadok et al., 1992a and b; Martin et al., 1995). PS is an anionic phospholipid normally confined to the inner leaflet of the membrane bilayer (Op den Kamp, 1979) by an ATP-dependent amino phospholipid translocase (Verhoven et al., 1995; Tang et al., 1996). The mechanisms mediating PS exposure are complex but seem to involve both loss of amino phospholipid translocase activity and activation of a non-specific lipid scramblase (Verhoven et al., 1995; Bratton et al., 1997).

Annexin V, a family member of the calcium and phospholipid binding proteins that are expressed in all mammalian cells except red blood cells (Raynal and Pollard, 1994), has been shown to preferentially bind PS and to inhibit PS-dependent procoagulant reactions (Tait et al., 1989; Andree et al., 1990). It has 320 amino acids with a MW of 35.8 kDa (Grundmann et al., 1988). Although its biological functions *in vivo* remain unknown, *in vitro* experiments revealed several properties including inhibition of phospholipase A_2, anticoagulation and formation of ion channels (reviewed by Raynal and Polland, 1994; Moss, 1997). The utility of annexin V in flow cytometry applications is derived from its selective affinity for negatively charged phospholipids. Under defined salt and calcium concentrations, annexin V has specificity for PS over other phospholipid species, with K_d for its binding to PS estimated at 2×10^{-10} M (Andree et al., 1990). The stoichiometry of annexin V binding to PS ranges between 4 and 8 annexin V molecules per one PS molecule (Meers and Mealy, 1993; Pigault et al., 1994). Based on these properties, a flow cytometric method was developed using FITC-labeled annexin V to bind PS on the outer leaflet of plasma membrane for quantifying apoptotic cells (Koopman et al., 1994; Martin et al., 1995b).

Briefly, 0.5 x 10^6 cells were pelleted at 200g for 4 min and re-suspended in 1 ml Hepes-buffered saline (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and incubated with 2.5µl FITC- labelled annexin V (Bender MedSystems, Vienna, Austria) for 8 min at room temperature in the dark. After incubation, 2.5 µg PI was added and the sample was analysed in a Becton Dickinson FACScan flow cytometer equipped with an Argon laser emitting excitation light at 488 nm. The green fluorescence (520 nm) generated from FITC conjugated annexin V and red fluorescence resulting from PI were recorded, both with logarithmic amplification. Cells, which included PI, were considered non-viable and gated-out. The percentage of the remaining, viable cells with high green fluorescence was then determined as apoptotic cells.

2.4.4 Measuring $\Delta\Psi_m$ using the fluorescent dye, $DiOC_6(3)$

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by incubating cells (0.5 x 10^6 /ml) for 20 min at 37°C with the cationic lipophilic fluorochrome, 3,3'dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 50 nM) (Molecular Probes Inc., Eugene, OR) which is retained in mitochondria with a normal membrane potential (Petit et al., 1990). Control experiments were performed by incubating untreated cells for a further 10 min at 37°C with carbonyl cyanide m-chlorophenyl hydrazone (m-ClCCP, 50 µM,), an uncoupling agent which abolishes the $\Delta\Psi_m$. At the end of incubation, 2.5 µg PI was added and the sample was analysed in a Becton Dickinson FACScan flow cytometer equipped with an Argon laser emitting excitation light at 488 nm. The green fluorescence (520 nm) generated from $DiOC_6(3)$ and red fluorescence resulting from PI were both recorded with logarithmic amplification. Cells with PI inclusion were considered non-viable and gated-out. The percentage of the remaining, viable cells with low green fluorescence (below the threshold set up by using the mitochondrial uncoupler as a positive control) was then determined as cells with decreased $\Delta\Psi_m$. Figure 3 was an example of the flow cytometric analysis of the $\Delta\Psi_m$ after staining with $DiOC_6(3)$.

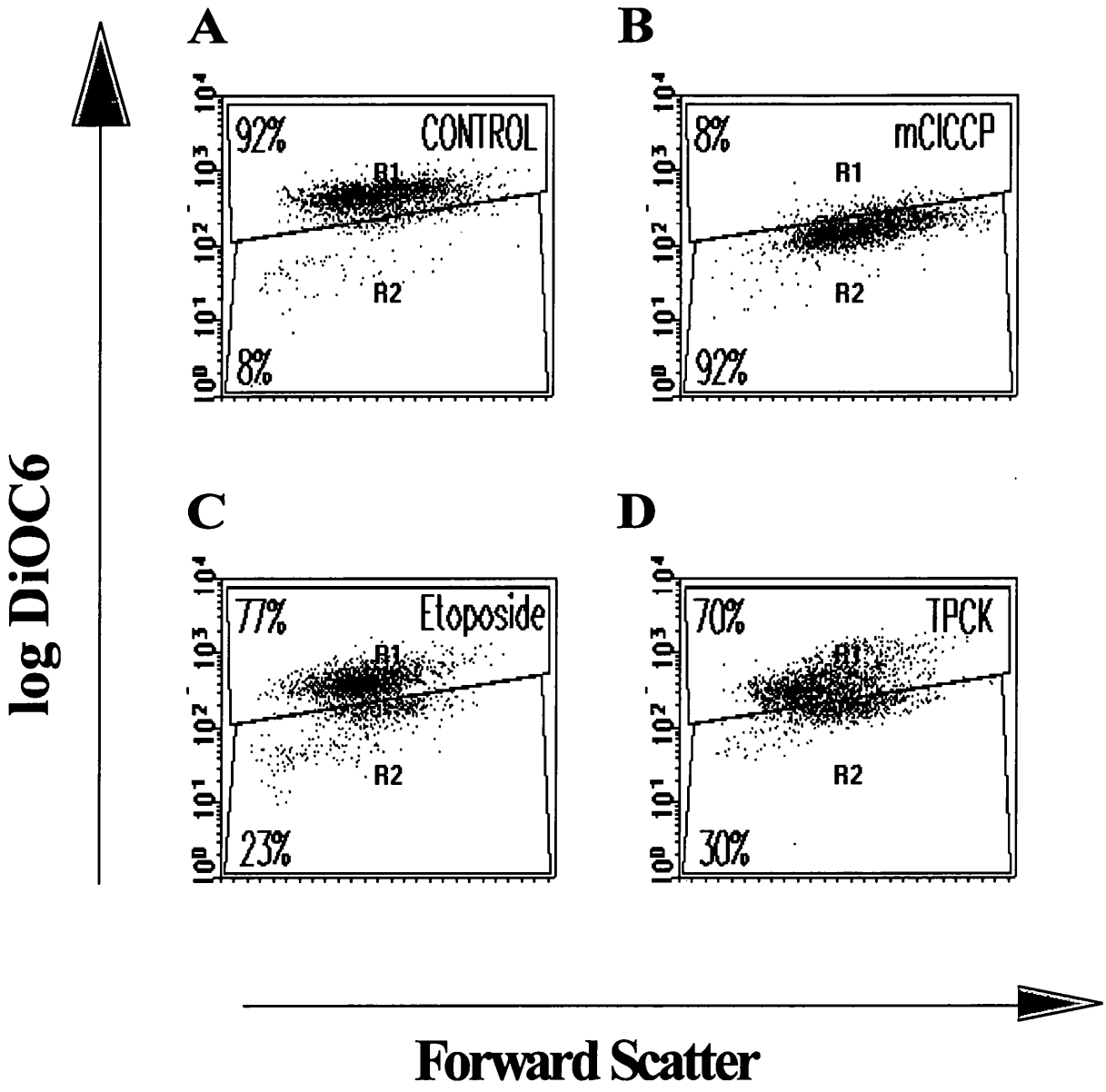


Figure 3. Flow cytometric analysis of the $\Delta\Psi_m$ after staining with $DiOC_6(3)$

The majority of control cells have high mitochondrial $\Delta\Psi_m$ (A). Apoptotic stimuli induced a decrease in mitochondrial $\Delta\Psi_m$ (C and D). Carbonylcyanide m-chlorophenylhydrazone (mClCCP, 50 μM), a mitochondrial uncoupler, was used as a positive control (B).

2.5 Analysis of caspase activation and proteolytic activity

2.5.1 Introduction

Caspases are cysteine proteases, which have an absolute specificity for an aspartic acid in the P_1 position of the substrate (Thornberry et al., 1992). They exist in cells as inactive proenzymes. Upon activation, the procaspases are cleaved at specific aspartate residues, generating a large and small subunit, which together form the

active enzyme (Thornberry and Molineaux, 1995; Kumar and Lavin, 1996; Cohen, 1997). Thus, western blot analysis using antibodies raised against either the large or small subunit of a particular caspase has become a commonly used method to measure the activation/processing of the caspase during the induction of apoptosis. Once activated, caspases cleave many intracellular proteins including lamins and PARP. Although the precise substrate specificity of most caspases is unknown, PARP is cleaved at a DEVD↓G sequence by caspases-3 and -7 (Lazebnik et al., 1994; Fernandes-Alnemri et al., 1995b), whereas lamins are preferentially cleaved at a VEID↓N sequence by caspase-6 (Orth et al., 1996; Takahashi et al., 1996). Therefore, the proteolytic activity of particular caspase(s) can be measured fluorimetrically using an appropriate substrate. Again, western blot analysis using antibodies recognising the respective fragments of the intracellular proteins known to be cleaved by caspases during apoptosis is a useful tool for measuring the proteolytic activity of caspase(s).

2.5.2 Fluorimetric measurement

2.5.2.1 Preparation of cytosolic extracts

Isolation of the cytosolic extracts was essentially as described (Bossy-Wetzel et al., 1998). Briefly, 50 x 10^6 THP.1 cells were washed twice in ice-cold PBS, pH 7.4 and centrifuged at 200g for 5 min. The cell pellet was re-suspended in 200 µl of extraction buffer (220 mM mannitol, 68 mM sucrose, 50 mM Pipes/KOH, pH 7.5, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin A and 10 µg/ml aprotinin). After 30 min incubation on ice, cells were homogenised using a glass Dounce homogeniser, with 40 strokes of the "B" pestle. The homogenates were then centrifuged at 14,000g, for 15 min at 4°C, and the resulting supernatants were used as the cytosolic extracts for measuring protease activity and stored at -80 °C. The protein concentration of the cytosolic extracts was determined by the Bradford assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, England).

2.5.2.2 Enzymic assay

The proteolytic activity of caspase(s) in cell lysates was measured using a continuous fluorimetric assay initially described by Thornberry (1994), with minor modifications

(MacFarlane et al., 1997). Liberation of fluorogenic 7-amino-4-trifluoromethylcoumarin from Ac-DEVD.AFC (Enzyme Systems Inc.) as a measure of caspase-3 like activity was assayed at excitation and emission wavelengths of 400 and 505 nm, respectively. Lysates were assayed at 37°C in a modified thermostatted cuvette holder in 1.25 ml of 100 mM Hepes, pH 7.5, 10 % sucrose, 0.1 % CHAPS, 10 mM dithiothreitol in an LS50B luminescence fluorimeter (Perkin-Elmer Corp., Norwalk, CT). Routinely, the assays contained 50 or 100 μg of lysate protein and 20 μM substrate. Calibration was carried out with a standard solution of 7-amino-4-trifluoromethylcoumarin (0-1.6 μM). The protease activity was expressed as pmol AFC/mg protein/min.

2.5.3 Western blot analysis

2.5.3.1 SDS-PAGE

SDS-PAGE was performed essentially as described (Harlow and Lane, 1988). Briefly, 0.2 x 10^6 cells were prepared and lysed in sample buffer (62.5 mM Tris/HCl, pH 6.8, 15 % glycerol, 2 % SDS, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 % β-mercaptoethanol and 0.005 % bromophenol blue). To assist the dissolution of DNA, 4 M Urea was added to the sample buffer and samples were sonicated on ice. Cellular proteins were then denatured by boiling for 4 min and resolved on SDS-polyacrylamide gels using the Mini-PROTEAN II electrophoresis cell (Bio-Rad) with the electrophoresis run at 140 volts for approximately an hour.

2.5.3.2 Immunoblotting

After electrophoresis, proteins were transferred onto nitrocellulose membrane (Hybond-ECL, Amersham, Little Chalfont, England) at 4°C with the electrophoresis run at either 30 volts overnight or 100 volts for one hour using the Mini Trans-Blot cell (Bio-Rad). After blocking the non specific binding sites with 5 % non-fat milk in TBS-T (Tris buffered saline containing 20 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1 % Tween-20), membranes were probed with appropriate antibodies. Intact PARP and its 85 kDa fragment were detected using rabbit antiserum (318) (diluted at 1:8,000 in TBS-T) or mouse monoclonal antibody (C2-10) at a dilution of 1:10,000 (both provided by Dr. G.G Poirier, Laval University, Quebec, Canada). Intact lamin B1 and its proteolytic fragment were detected with a mouse monoclonal antibody (diluted at

1:50) (Serotec Ltd., Oxford, England). Pro-caspase-3 and its catalytically active large subunit (p17 fragment) were detected using a rabbit polyclonal antibody directed to the p17 fragment at a dilution of 1:10,000 (provided by Dr. D. Nicholson, Merck Frosst, Quebec, Canada). Pro-caspase-7 and its catalytically active large subunit (p19 fragment) were detected using a rabbit polyclonal antibody as described (MacFarlane et al., 1997). Intact Bid and its cleaved products were detected using an antibody provided by Dr. X. Wang as described (Luo et al., 1998). Final detection was achieved using the appropriate secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG) conjugated with horseradish peroxidase (HRP) together with an enhanced chemiluminescence (ECL) kit (Amersham).

For detecting cytochrome c, 30 μg of protein from the cytosolic extracts prepared as described in section 2.5.2.1 was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with a mouse monoclonal antibody recognising human cytochrome c (7H8.2C12) (PharMingen, San Diego, CA) at a dilution of 1:2,000. Final detection was as described above. Parallel samples, containing an equal amount of cytosolic protein, were checked by Western blotting for mitochondrial contamination of the cytosolic extracts using the 12C4-F12 mouse monoclonal antibody (Molecular Probes) to mitochondrial membrane-bound cytochrome c oxidase (subunit II) as described by Kluck et al. (1997).

2.6 Analysis of DNA fragmentation

2.6.1 Field inversion gel electrophoresis

Field inversion gel electrophoresis (FIGE) was used to detect large kilobase pair DNA fragments as described (Brown et al., 1993). Agarose plugs containing 0.5 x 10^6 cells were prepared and digested with Pronase (1 mg/ml, Boehringer-Mannheim UK) for 48 h at 50°C in a buffer containing 1 % lauryl sarcosine, 0.5 M EDTA, and 10 mM Tris (pH 9.5). Electrophoresis was carried out using a vertical gel chamber at 200 volts starting with a 15 min continuous forward pulse followed by a 2.4 second forward to 0.8 second reverse (3:1 ratio) pulse produced by a PC 750 pulse controller (Hoefer Scientific Instruments, San Francisco, CA, USA) for 1 h. Then, a ramp factor of 1.5 was applied to increase the forward pulse time to 24 second and reverse phase to 8 second, and the gel was run for a further 6 h. Electrophoresis buffer was 0.5 x

TBE (89 mM Tris, 50 mM boric acid and 2.5 mM EDTA) at pH 8.2. Two sets of standards were used: *Saccharomytes cerevisiae* chromosomes (243-2200 kilobase pairs from Clontech (Cambridge, UK) and 0.1-200 kilobase pairs (Sigma). At the end of the electrophoresis, the gel was rinsed in de-ionised water and stained with ethidium bromide (1 μg/ml) for 30 min and the DNA was visualised under UV light.

2.6.2 Agarose gel electrophoresis

Conventional agarose gel electrophoresis was used to detect internucleosomal fragments of DNA, as described (Sorenson et al., 1990). A 1.8 % agarose gel in 0.5 x TBE buffer was prepared as a resolving gel. A digestion gel (0.8 % agarose, 2.5 % SDS in 0.5 x TBE buffer) was also prepared to ensure that DNA from intact cells can enter the gel, together with the addition of 1.25 mg/ml Proteinase K, which was added when the gel has cooled to just below 50°C. The digestion gel was quickly poured into a space above the wells previously cut in the resolving gel. 2 $x10^6$ cells were pelleted and resuspended in 20 μl of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) to which 7 μl of RNase (50 mg/ml) was added. The cells were digested for 20 min at room temperature before mixing with 5 μl of loading buffer (Sigma) and loaded into the wells. 123 base pair DNA ladders (Life Technologies Ltd.) were used as standards. Electrophoresis was run in 0.5 x TBE buffer at 20 volts for one hour, followed by a further four hours at 100 volts. The DNA fragments were visualised as described above.

2.7 Phagocytosis assay

Isolation and culture of mouse macrophages were essentially as described (Fadok et al., 1992a) except for minor modifications as follows. Inflammatory macrophages were elicited into the peritoneal cavity of 8- to 16-week old Balb/c mice with 2% Brewer's thioglycollate. Peritoneal exudate cells were harvested 4 days later by peritoneal lavage with balanced salt solution, plated in 96-well plates at $0.1x10^6$ cells per well and cultured overnight in complete medium (DMEM containing 5% FCS, 50 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin) before use in the phagocytosis assay. Bone marrow was harvested from Balb/c mice and plated at $0.1x10^6$ cells per well in 96-well plates in DMEM containing 10% conditioned medium from L929 cells as a source of macrophage-CSF.

Bone marrow macrophages were used in the phagocytosis assay after being cultured for 5 to 7 days. For the phagocytosis assays, macrophage monolayers were washed and THP.1 cells (0.5×10^6) in RPMI 1640 containing 5 % FCS added to each well. The plates were incubated for 1 h, then washed in ice-cold saline after treatment with trypsin to remove adherent macrophages. After cytospinning the macrophages onto microscope slides, cells were fixed with 2 % glutaraldehyde and stained with Giemsa dye. Phagocytosis was evaluated by counting 500 macrophages per slide of four replicate slides in each experiment. Results were expressed as the percentage of macrophages that had phagocytosed THP.1 cells.

2.8 ATP measurement

Intracellular ATP was determined luminometrically using a bioluminescent somatic cell assay kit (Sigma) according to the manufacturer's instructions. In principle, ATP released from cells is utilised for the oxidation of firefly luciferin by O_2 in the presence of Mg^{++}. This reaction, mediated by firefly luciferase, results in light emission (λ_{max} ~565 nm) (Anthony et al., 1985). The amount of light emitted was then measured on a Wallac 1450 Microbeta Liquid Scintillation Counter (Wallac OY, Turku, Finland).

Chapter 3

Characterisation of the major changes in chemical-induced apoptosis

3.1 Introduction

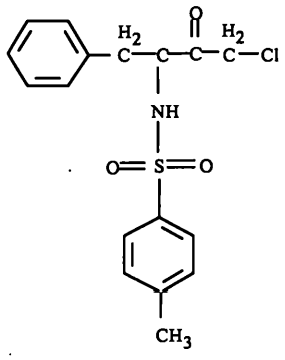
Apoptosis is a fundamental form of cell death which plays a major role in the development and homeostasis of multicellular organisms (Arends and Wyllie, 1991). Disturbances in apoptosis are implicated in cancer, acquired immunodeficiency syndrome and some neurodegenerative disorders (Thompson, 1995). Apoptosis is also an important form of chemical-induced cell death (Corcoran et al., 1994). Apoptotic cell death comprises an initial commitment phase followed by an execution phase, which is characterised by a consistent series of distinct morphological changes (Earnshaw, 1995), suggesting the existence of a common execution machinery (Jacobson et al., 1994). In the nematode *C. elegans*, the gene *ced-3* encodes a protein required for developmental cell death (Ellis et al., 1991). Since the recognition that CED-3 has sequence homology to the mammalian cysteine protease, interleukin-1β converting enzyme (ICE) (Yuan et al., 1993b), a family of at least thirteen related ICE-like proteases, now known as caspases (Alnemri et al., 1996), has been identified. Caspases are characterised by an absolute specificity for aspartic acid in the P_1 position of the substrate (reviewed by Thornberry and Molineaux, 1995; Kumar and Lavin, 1996; Cohen, 1997). They exist in cells as inactive proenzymes and, upon activation, are cleaved at specific aspartate residues, generating a large and a small subunit, which together form the active enzyme in the form of a heterotetramer containing two large and two small subunits (Walker et al., 1994; Wilson et al., 1994). Caspases may be divided into "initiator" caspases with long prodomains (e.g. caspase-8, -9 and -10), which activate "effector" caspases with short prodomains (e.g. caspase-3, -6 and -7), which in turn cleave intracellular proteins, such as poly (ADP-ribose) polymerase (PARP) and lamins, so precipitating the dramatic morphological and biochemical changes of apoptosis (Cohen, 1997). Thus, caspases appear to play a critical role during the execution phase of apoptosis.

Reduction in mitochondrial membrane potential ($\Delta\psi_m$) has also been observed to occur early during the induction of apoptosis by a variety of stimuli (Zamzami et al., 1995; Castedo et al., 1996) and possibly to regulate the aberrant exposure of phosphatidylserine (PS) on the outer plasma membrane of the cells undergoing apoptosis (Castedo et al., 1996). The decrease in $\Delta\psi_m$ probably results from the opening of the permeability transition (PT) pore or megachannel in the inner

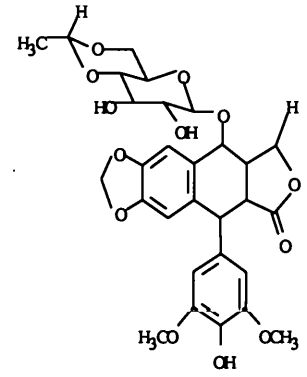
mitochondrial membrane (reviewed by Susin et al., 1996a). The induction of PT in isolated mitochondria results in the release of a factor capable of causing isolated nuclei to undergo chromatin condensation and DNA fragmentation (Marchetti et al., 1996b). Inhibition of PT by pharmacological agents or hyperexpression of Bcl-2 prevented apoptosis (Susin et al., 1996a and 1997) and so mitochondrial permeability transition is proposed as a central coordinating event of apoptosis (Kroemer et al., 1997).

The objective of the present study is to characterise the biochemical and morphological features in chemical-induced apoptosis and to investigate the temporal relationship of the biochemical events leading to these features using an established cell system, human monocytic THP.1 cell line. Two chemicals with different modes of action were used to induce apoptosis, i.e. etoposide, and N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK). TPCK is a chymotrypsin-like serine protease inhibitor (Fig. 4). The chloromethyl ketone in the compound functions as an reactive group, which alkylates one of the two nitrogen atoms of the imidazole ring of histidine 57 of the protease, while its phenylalanyl side chain enables TPCK to bind specifically to chymotrypsin, resulting in the irreversible inhibition of the enzymic activity (Shaw, 1970). Because of this property, TPCK was developed as one of the affinity-labelling reagents for the study of the catalytic role of individual residues in functionally-related enzymes. It has also been used to study many biological processes including fertilization, cell growth, protein synthesis, and diseases such as cancer (reviewed by Powers, 1977). However, possibly due to the multiple functions of the chloromethyl ketone group, TPCK was shown to inhibit many functionally unrelated enzymes such as cAMP-dependent protein kinase (Kupfer et al., 1979) and protein kinase C (Solomon et al., 1985), and also to block the activation of nuclear factor (NF)-kappaB (Henkel et al., 1993). Recently, TPCK and many other protease inhibitors were used to investigate the role of proteases in apoptosis. TPCK has been shown to inhibit apoptosis as assessed by internucleosomal DNA fragmentation (Bruno et al., 1992; Weaver et al., 1993). However, studies carried out in this laboratory demonstrated that TPCK alone induced both biochemical and morphological changes associated with early nuclear changes of apoptosis in thymocytes without the formation of internucleosomal DNA fragments (Fearnhead et al.,1995b). This initial observation

was later confirmed in THP.1 cells (Zhu et al., 1997). TPCK was also reported to induce apoptosis in HL-60 cells in the absence of DNA ladder formation (Lu and Mellgren, 1996). Thus the target(s) of TPCK may not only be engaged in the terminal stage of the execution phase, involving internucleosomal DNA fragmentation but also in the regulation of apoptosis at a much earlier stage.



TPCK



Etoposide

Figure 4. The structures of TPCK and etoposide

Etoposide (Fig. 4), a semisynthetic derivative of the naturally occurring antimitotic agent podophyllotoxin, has been widely used as an anti-cancer drug (reviewed by Imbert, 1998). It is believed to act as a specific poison for the cell cycle-regulated protein DNA topoisomerase II (Ross et al., 1984). Topoisomerase II is an enzyme that unknots, decatenates or relaxes the supercoiled DNA molecules by introducing transient double-strand breaks through which the strands of an intact helix can pass (reviewed by Berger and Wang, 1996). In order to maintain the integrity of the cleaved genetic material during this process, the enzyme forms a proteinaceous bridge that spans the nucleic acid break. This bridge is anchored by a covalent phosphotyrosyl bond between the active site residues of the homodimeric enzyme and the newly created 5'-DNA termini, forming an enzyme-cleaved DNA complex (also known as a cleavage complex) (Froelich-Ammon and Osheroff, 1995). As the process of forming this cleavage complex is required in a number of critical nuclear processes including DNA replication and recombination, as well as chromosome segregation (reviewed by Corbett and Osheroff, 1993), topoisomerase II is essential for the survival of proliferating eukaryotic cells. Normally, the topoisomerase II-

cleaved DNA complex is a short-lived intermediate in the catalytic cycle of the enzyme and is therefore tolerated by the cell. However, when present in high concentrations, the cleavage complexes become potentially toxic, promoting permanent double stranded DNA breaks. Etoposide interacts directly with topoisomerase II (rather than DNA) and it is this interaction which mediates the drug-induced formation of enzyme-DNA cleavage complexes and inhibits DNA religation (Burden et al., 1996). DNA is damaged as a result of the formation of permanent double stranded breaks. Etoposide has been shown to induce apoptosis in various cell systems including human HL-60 and KG1A leukaemic cells (Kaufmann, 1989), human colon cancer cell lines HT-29 and COLO 320 (Bertrand et al., 1991), human leukaemic U937 cells (Mashima et al., 1995), human breast carcinoma cell line MCF7 (Benjamin et al., 1998), and primary rat thymocytes (Walker et al., 1991; Sun et al., 1992), as well as human monocytic THP.1 cells (Zhu et al., 1995).

3.2 Morphological characterisation of apoptosis

Although both TPCK and etoposide have been shown to induce apoptosis in THP.1 cells, the biochemical and morphological changes have not been fully characterised. In particular the relationships between these changes have not been adequately investigated. In the present study, several criteria were used to assess apoptosis in an attempt to detect different characteristics of the apoptotic phenotype yet with the morphological changes as the prime criterion.

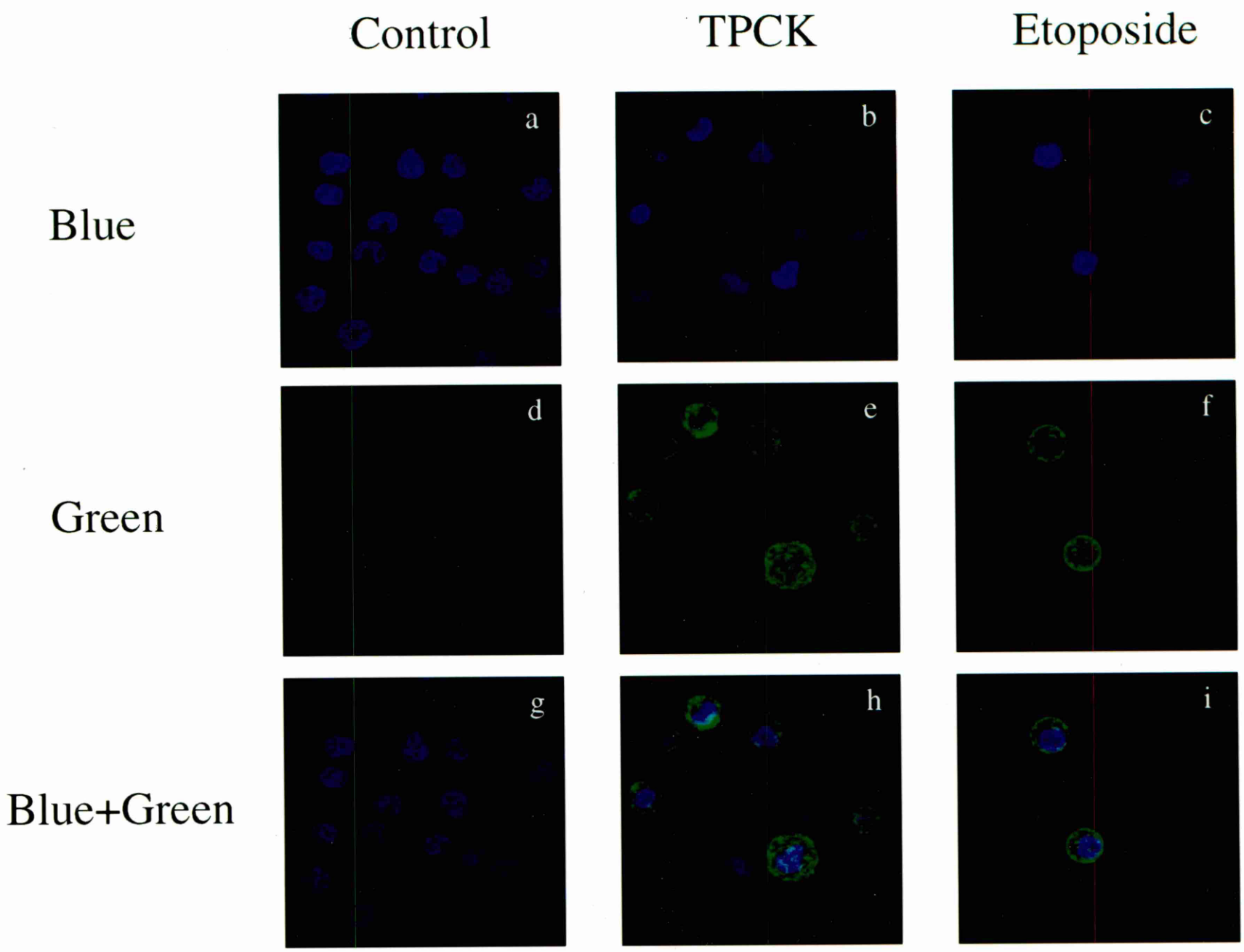
3.2.1 Nuclear condensation and fragmentation

It has been shown that incubation of human monocytic THP.1 cells with diverse stimuli, including etoposide, induced apoptotic cells. Condensed and fragmented nuclei are visible by fluorescence microscopy when these cells are stained with the membrane permeable DNA binding dye, Hoechst 33342 (Zhu, 1995). Externalisation of PS is a general feature of apoptosis, which can be detected using the PS-specific binding protein annexin V, labelled with FITC (Tait et al., 1989; Andree et al., 1990). Treated cells were therefore examined by confocal microscopy after Hoechst 33342 staining and FITC-labelled annexin V binding. This technique allowed the simultaneous analysis of both nuclear condensation and fragmentation (blue fluorescence from Hoechst 33342) and externalised PS (green fluorescence from

FITC). Treatment with either TPCK (75μM) or etoposide (25 μM) resulted in the induction of apoptotic cells with condensed nuclei which fluoresced more brightly than control cells (Fig. 5 compare b and c with a). This increase in Hoechst fluoresence resulted from an increased cell permeability of apoptotic cells compared with normal cells (Ormerod et al., 1993). The nuclei of the cells treated with either TPCK or etoposide also became fragmented (Fig. 5b and c), exhibiting a distinct apoptotic morphology. Incubation of cells with either TPCK or etoposide also resulted in the increase in green fluorescence in the plasma membrane, indicating the binding of annexin V to PS (Fig. 5e and f, respectively), whereas untreated control cells showed no increase in green fluorescence (Fig. 5d). Combined image (blue + green fluorescence) showed that cells with condensed and fragmented nuclei also exhibited a concomitant increase in green fluorescence (Fig. 5h and i). Thus, nuclear condensation and fragmentation were co-localised with externalised PS in cells treated with either TPCK or etoposide.

Figure 5. Confocal microscopy of co-localisation of nuclear condensation and fragmentation with PS externalisation

THP.1 cells were incubated for 4h either alone or with TPCK (75 μM) or with etoposide (25 μM) and examined by confocal microscopy after Hoechst 33342 staining (Blue) and FITC-labelled annexin V binding (Green). Combined image (Blue + Green) showed that cells with condensed and fragmented nuclei exhibited a concomitant increase in green fluorescence. Cells were viewed with a x100 objective.

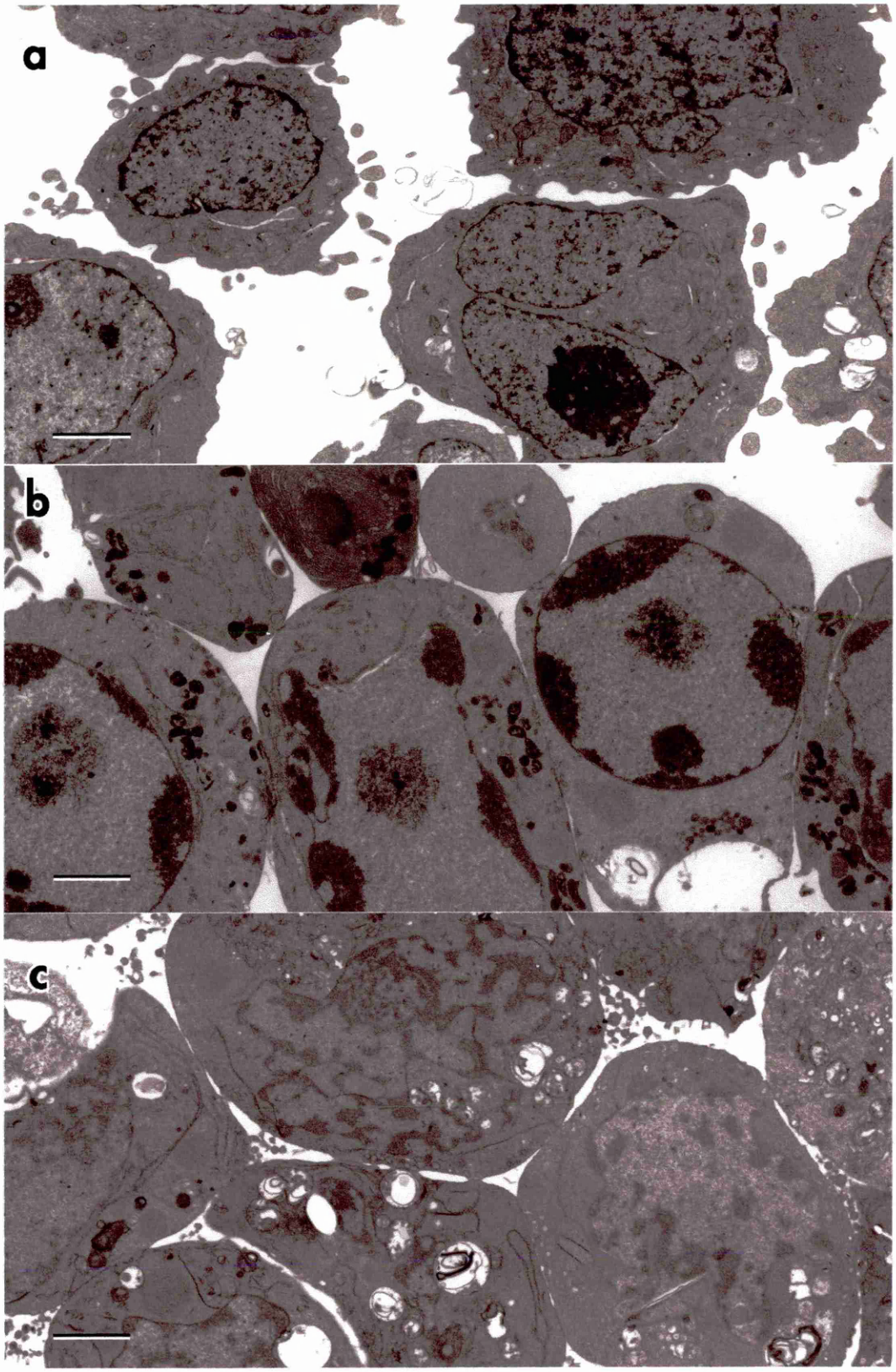

Control
TPCK
Etoposide
Blue
Green
Blue+Green
a
b
c
d
e
f
g
h
i

3.2.2 Chromatin condensation

The ultrastructure of the treated cells was examined in order to see if they exhibit the classical morphology of apoptosis. Untreated THP.1 cells appeared to be irregular in outline with many microvilli and a multi-lobed nucleus (Fig. 6a). Cells treated with etoposide exhibited an increase in cytoplasmic density and chromatin condensation into crescentic accumulations against the inner membrane of the nuclear envelope (Fig. 6b). These changes were consistent with the characteristic ultrastructural features of apoptosis initially described (Kerr et al., 1972). Cells treated with TPCK also showed cytoplasmic condensation accompanied by pronounced dilation of the endoplasmic reticulum, with the resulting vacuoles showing signs of fusion with the cell membrane, usually at one pole of the cell (Fig. 6c). The membrane of treated cells was largely devoid of microvilli and the cytoplasm contained small accumulations of fine granular material. The cytoplasmic changes brought about by TPCK treatment were similar to those resulting from etoposide treatment. The degree of chromatin condensation was intermediate between control cells (Fig. 6a) and etoposide-treated cells (Fig. 6b), in agreement with a previous study (Zhu et al., 1997). Thus, incubation of THP.1 cells for 4 h with either etoposide or TPCK induced apoptosis, characterised morphologically by both cytoplasmic and nuclear changes.

Figure 6. Etoposide and TPCK induced ultrastructural changes of apoptosis

Induction of apoptosis was as described in legend to figure 5. (a) Control THP.1 cells following incubation for 4 h in control medium showed normal ultrastructure. (b) Cells incubated with etoposide (25 μM) showed perinuclear accumulations of condensed chromatin. (c) Cells treated with TPCK (75 μM) exhibited dilated endoplasmic reticulum and the accumulation of fine granular material with many small clumps of partially condensed chromatin throughout the nucleoplasm [all bar=2.5 μm]


a
b
c

3.3 DNA fragmentation

The nuclear alterations have often been shown to be associated with the fragmentation of DNA. Initially, DNA is cleaved to 200-300 and 30-50 kilobase sized fragments, which are then further degraded to form internucleosomal fragments (Brown et al., 1993; Cohen et al., 1994). The latter fragments, demonstrated as a DNA ladder by agarose gel electrophoresis, were originally considered as a biochemical hallmark of apoptosis (Wyllie, 1980). The formation of internucleosome-sized DNA fragments is now considered as a late event in apoptosis, which may be dissociated from early more critical changes (G. Cohen et al., 1992). The effect of two proapoptotic stimuli on DNA fragmentation was also investigated in the present study. Incubation of THP.1 cells for 4 h with TPCK induced fragmentation of DNA to large fragments of 200-300 and 600-700 kilobase pairs in length detected by field inversion gel electrophoresis (Fig. 7 lane 2). Treatment with etoposide also resulted in the formation of large kilobase pair fragments of DNA, but mainly of 30-50 kilobase pair in length (Fig. 7 lane 3). No fragments were observed in untreated control cells (Fig. 7 lane 1). Etoposide also induced the typical ladders of internucleosomal DNA fragments (see Fig. 13 in Chapter 4). The effect of TPCK on internucleosomal cleavage of DNA in THP.1 cells has been previously investigated (Zhu et al., 1997). TPCK, at the concentrations (50-75 μM) which induced apoptosis, did not induce internucleosomal cleavage of DNA. However, a lower concentration of TPCK (10 μM) inhibited the internucleosomal cleavage of DNA induced by many other apoptotic stimuli including etoposide (Zhu et al., 1997). Thus, induction of apoptosis by TPCK was accompanied by the formation of large kilobase pair fragments of DNA, but without internucleosomal fragments, whereas in etoposide-induced apoptosis both large kilobase pair and internucleosomal fragments of DNA were observed, in agreement with previous observations (Zhu et al., 1995 and 1997).

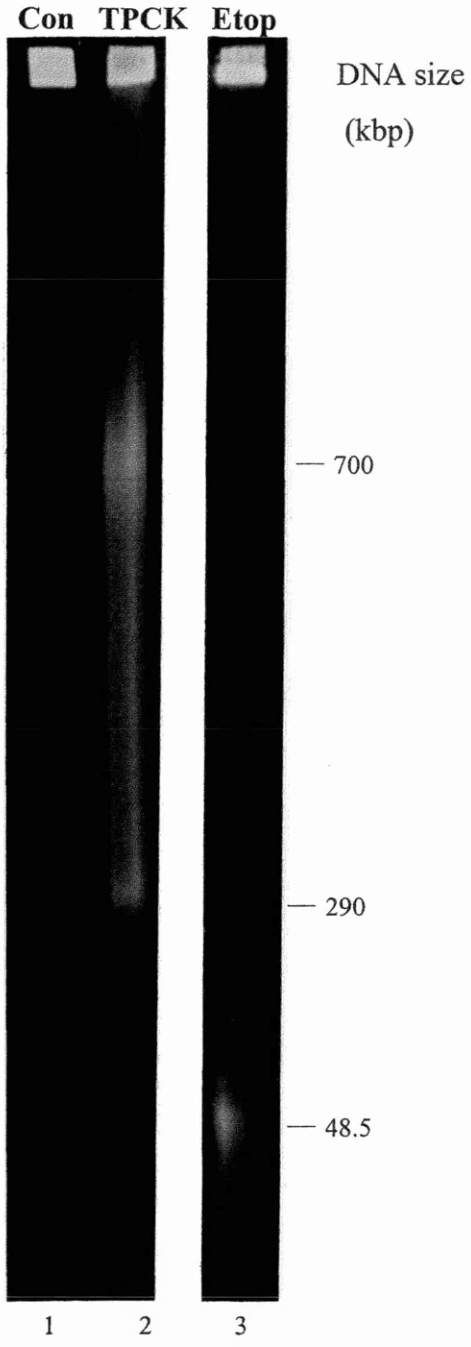


Figure 7. Apoptosis induced by TPCK or etoposide is accompanied by the formation of large kilobase pair fragments of DNA.

Cells were incubated for 4h either alone (Con., lane 1) or with 75 μM TPCK (lane 2) or with 25 μM etoposide (Etop., lane 3) and examined for large kilobase pair (kbp) fragments of DNA by field inversion gel eletrophoresis as described in Materials and Methods.

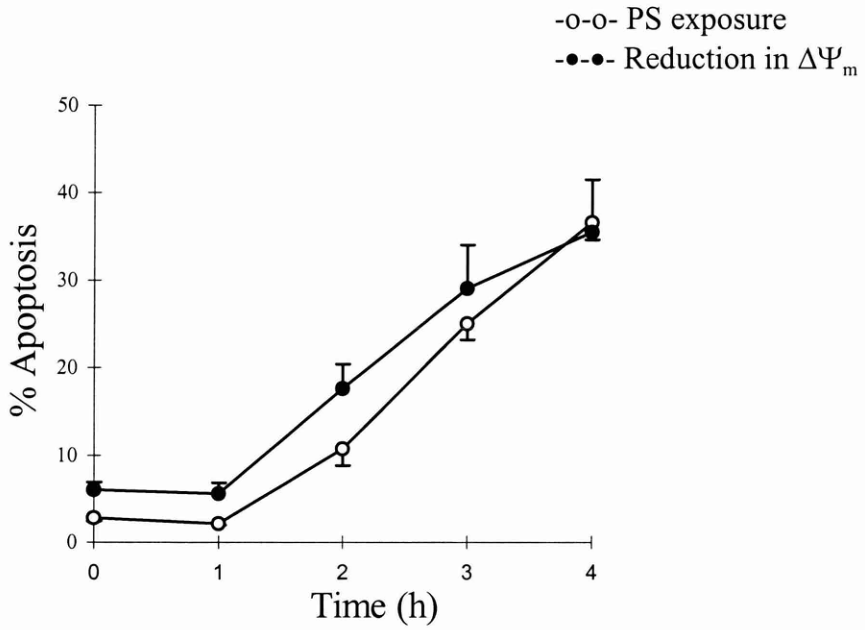
3.4 PS externalisation and reduction in $\Delta\Psi_m$

As described earlier, exposure of PS is a general phenomenon of apoptosis which can be detected by binding of FITC-labelled annexin V and a flow cytometric method has been developed to quantify these cells (Koopman et al., 1994; Martin et al., 1995). In addition, a reduction in $\Delta\Psi_m$ has also been reported to be an early event in the induction of apoptosis in many different systems (Zamzami et al., 1995; Castedo et al., 1996). Using the membrane potential-sensitive fluorescent dye, $DiOC_6(3)$, changes in mitochondrial membrane potential can be measured by flow cytometry (Petit et al., 1990). Thus, in the present study, apoptosis was quantitated primarily by these two flow cytometric methods, measuring the percentage of cells with externalised PS and decreased $\Delta\Psi_m$. Incubation of THP.1 cells with either etoposide or TPCK resulted in a time-dependent increase in cells with externalised PS and decreased $\Delta\Psi_m$ (Fig. 8A and B, respectively). Untreated control cells did not exhibit increases in externalised PS or decreased $\Delta\Psi_m$ after 4 h incubation (see below). While a decreased $\Delta\Psi_m$ has been proposed to regulate the externalisation of PS (Castedo et al., 1996), in the present studies both these changes demonstrated a similar time dependence, occurring at 2 h after treatment, irrespective of the apoptotic stimulus (Fig. 8). Thus induction of apoptosis in THP.1 cells is accompanied by simultaneous increases in cells with externalised PS and decreased $\Delta\Psi_m$.

3.5 Activation of caspases

The results described earlier have shown that incubation of THP.1 cells with either etoposide or TPCK resulted in the induction of apoptosis, characterised by a series of distinct morphological and biochemical alterations. This suggested that there may exist a common biochemical execution machinery during the execution phase of apoptosis which follows the initial commitment to cell death. In the nematode *Caenorhabditis elegans*, the gene *ced-3* encodes a protein which is essential for developmental cell death. The protein was later found to bear marked sequence similarity to, and identity with, mammalian cysteine protease interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993b). This finding, together with the observation that overexpression of ICE (now known as caspase-1) induces apoptosis (Miura et al., 1993), suggests that caspase(s) may play a key role in the execution

A. Etoposide (25 μM)



B. TPCK (75 μM)

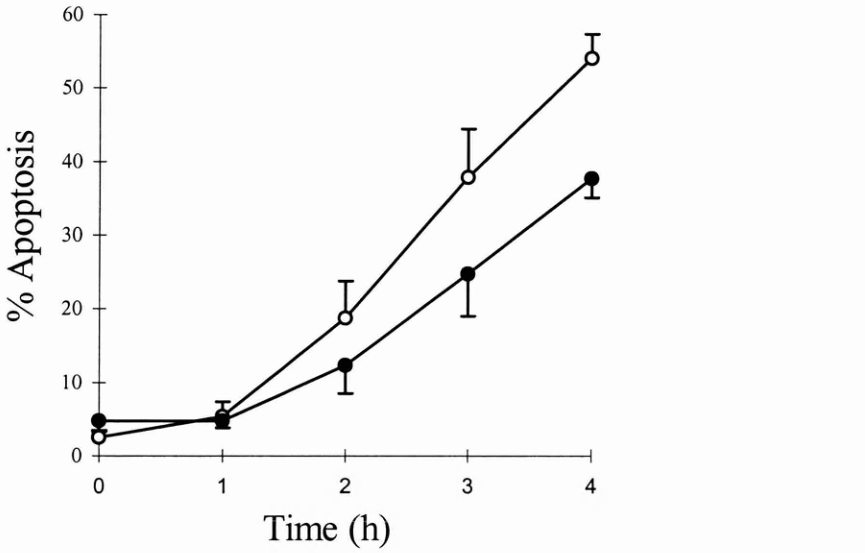


Figure 8. Time-dependent induction of apoptosis by etoposide and TPCK in THP.1 cells

THP.1 cells were incubated for up to 4 h with either etoposide or TPCK. The percentage of apoptotic cells was then determined flow cytometrically by the number of cells with externalisation of PS or a decrease in $\Delta\Psi_m$. The data represent the mean (± sem) of at least three experiments.

phase of apoptosis. It is therefore important to investigate the role of caspase(s) during the induction of apoptosis in this study.

3.5.1 Proteolytic activity of caspases

Caspases are cysteine proteases, which have an absolute specificity for an aspartic acid in the P_1 position of the substrate (Thornberry et al., 1992; Thornberry and Molineaux, 1995). In the present study, the proteolytic activity of caspases was measured by monitoring the Ac-DEVD.AFC cleavage activity of lysates from cells treated with etoposide or TPCK. The substrate Ac-DEVD.AFC was chosen because the DEVD tetrapeptide sequences mimic the cleavage site of intracellular protein PARP, i.e. $DEVD^{216}$-G^{217} (Lazebnik et al., 1994). In addition, caspase-3 (Nicholson et al., 1995), caspases-6 and -7 (Fernandes- Alnemri et al., 1995a and b) all have DEVD cleavage activity. The Ac-DEVD.AFC cleaving activity of lysates obtained from control cells was very low after 4 h incubation (Fig. 9). In contrast, incubation of THP.1 cells with either etoposide or TPCK resulted in a time-dependent increase in the Ac-DEVD.AFC cleavage activity (Fig. 9). In lysates prepared from etoposide-treated cells, an increase in Ac-DEVD.AFC cleavage activity was first observed at 2 h after treatment and continued to rise over a 4 h incubation period (Fig. 9), exhibiting a similar time-dependence to the induction of apoptosis as assessed by PS exposure and reduction in $\Delta\Psi_m$ (Fig. 8A). The increase in Ac-DEVD.AFC cleavage activity in lysates prepared from TPCK-treated cells was similar to that following etoposide treatment during the first 2 h of incubation, but then reached a plateau (Fig. 9). Marked differences were observed in both the level and pattern of proteolytic activity in lysates obtained from etoposide- and TPCK-treated cells. Whether the differences reflected distinct mechanisms employed by the two stimuli to induce apoptosis or were due to the inhibitory effects of TPCK on the proteolytic activity of activated caspases remains to be determined. An increase in Ac-DEVD.AFC cleavage activity was observed in lysates obtained from either etoposide- or TPCK-treated cells, thus providing evidence that caspases are involved in the induction of apoptosis.

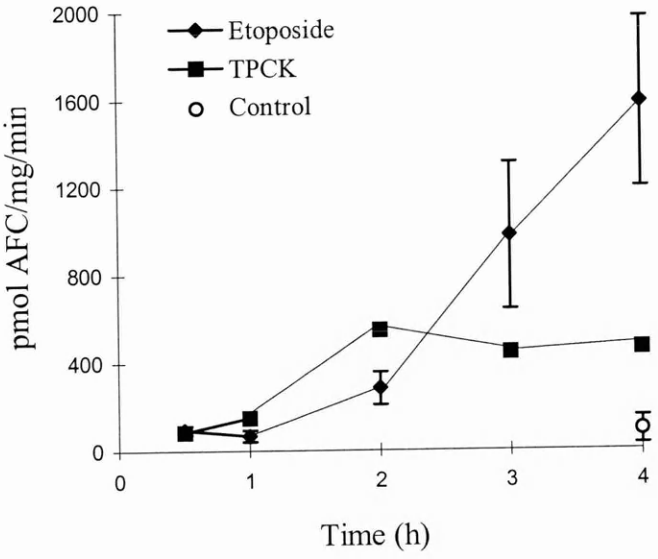


Figure 9. Ac-DEVD.AFC cleavage activity during the induction of apoptosis in THP.1 cells

THP.1 cells were incubated for up to 4 h either alone or in the presence of etoposide (25μM) or TPCK (75 μM) and lysates were prepared at the indicated time points as described in Materials and Methods. Ac-DEVD.AFC cleavage activity was measured as described in Materials and Methods.

3.5.2 Activation of caspases and cleavage of PARP

At least four caspases including caspases-2, -3, -6 and -7 are activated during the induction of apoptosis in THP.1 cells (MacFarlane et al., 1997; Zhu et al., 1997). However, the temporal relationship between activation of caspases and other features of apoptosis, such as reduction in $\Delta\Psi_m$ and PS exposure has not been investigated. Time course studies were carried out to examine the activation of caspases-3 and -7 and the proteolysis of the intracellular protein substrate PARP using Western blot analysis. Untreated control cells showed the presence of the intact ~32 kDa proform of caspase-3 and ~35 kDa proform of caspase-7 (Figs. 10A and B lane 1, respectively). Processing of caspase-3 at Asp^{175} between the large and small subunits yields a p20 fragment, which is further processed at Asp^{9} and Asp^{28} to yield p19 and p17 fragments, respectively (Nicholson et al., 1995; Fernandes-Alnemri et al., 1996). Processing of caspase-7 occurs at Asp^{198} between the large and small subunits, followed by cleavage at Asp^{23} to yield the p19 large subunit (Fernandes-Alnemri et al., 1995b). Induction of apoptosis with either etoposide or TPCK was accompanied by a time-dependent formation of the catalytically active large subunits of caspases-3

and -7 (Figs. 10A and B, lanes 2-9 respectively). The initial processing of caspase-7 occurs at 2 h in cells following incubation with etoposide (Fig. 10B lane 3) and at 1 h in cells treated with TPCK (Fig. 10B lane 6), whereas that of caspase-3 was barely detectable at 2 h in cells treated etoposide (Fig. 10A lane 3), but clearly detected in cells treated with TPCK (Fig. 10A lane 7). These results indicated that activation of caspase-7 may precede that of caspase-3. However, because the sensitivities of the antibodies used were unknown, caution should be exercised in the interpretation of the data. It is of interest to point out that dependent on the apoptotic stimulus, differences were observed in the formation of the large subunits of caspase-3 (Fig. 10A), possibly suggesting some differences in the mechanisms of activation. Induction of apoptosis by either etoposide or TPCK also resulted in a time-dependent loss of intact PARP together with formation of its characterisitic signature 85 kDa fragment (Fig. 10C). The fragment was first detected at 3 h after treatment with etoposide and at 2 h with TPCK treatment, apparently later than the activation of caspase-7 (Fig. 10B). Thus, induction of apoptosis in THP.1 cells was accompanied by cleavage of PARP and activation of caspases-3 and-7, providing further evidence of the involvement of caspases during apoptosis. The time-course studies indicated that caspase activation appeared to occur prior to PS exposure, reduction in $\Delta\Psi_m$, and cleavage of PARP.

3.6 Discussion

Treatment of THP.1 cells with either etoposide or TPCK resulted in the induction of apoptosis which was accompanied by an increasesd number of cells with externalised PS and decreased $\Delta\Psi_m$, DNA fragmentation, PARP cleavage and activation of caspases. These results suggested that, though the initial cell death inducing mechanisms may be different (see below), the two proapoptotic stimuli may utilise a similar execution machinery to bring about the morphological and biochemical features of apoptosis. The results from the time-course experiments suggested that caspases may be the common mediator in this model of apoptosis as caspase activation occurred prior to PS exposure and reduction in $\Delta\Psi_m$. However, caution must be exercised in the interpretation of these data because of the uncertainties in sensitivities of the different antibodies and between individual methods to assess apoptosis.

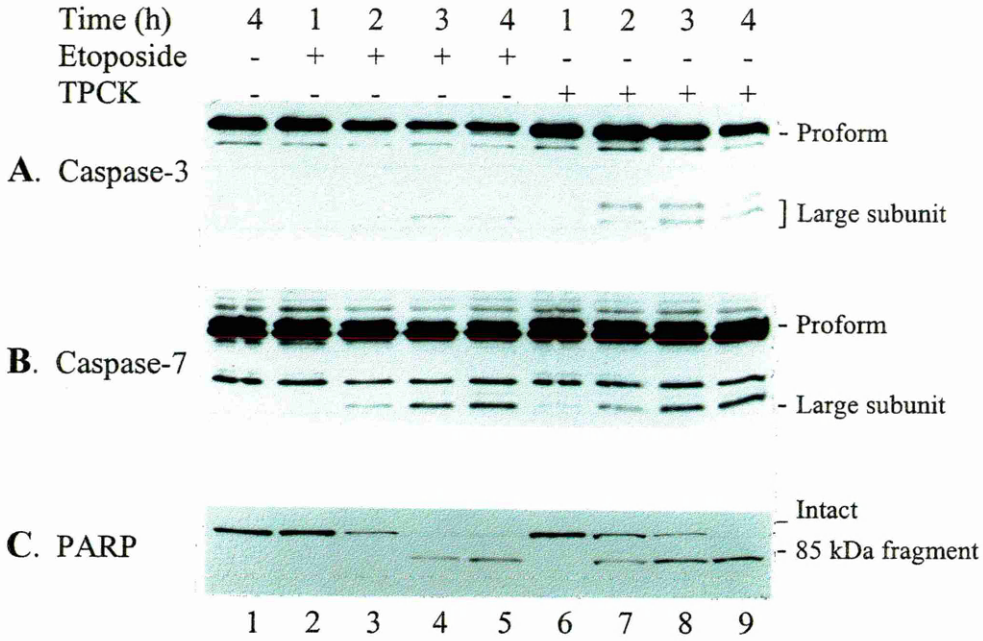


Figure 10. Apoptosis was accompanied by the activation of caspases-3 and -7 and cleavage of PARP

THP.1 cells were incubated for 4 h alone (lane 1), or up to 4 h with either etoposide (25 μM) (lanes 2-5) or TPCK (75 μM) (lanes 6-9). At the indicated time points, cells were analyzed by Western blot analysis for the cleavage of (A) 32 kDa pro-caspase-3, and (B) 35 kDa procaspase-7 to their respective catalytically active large subunits, as described in Materials and Methods. Cells were also analyzed using a mouse monoclonal antibody (C2-10) to detect both intact PARP and its cleaved 85 kDa fragment (C).

3.6.1 Mechanisms of apoptosis induced by TPCK

Although many features of apoptosis induced by the two proapoptotic stimuli appeared to be similar, marked differences were also observed particularly in the ultrastructure, formation of internucleosomal DNA fragments, Ac-DEVD.AFC cleavage activity in cell lysates and activation of caspase-3. Etoposide induced all the classical morphological and biochemical changes characteristic of apoptosis, whereas in TPCK-treated cells the degree of chromatin condensation was intermediate between control and etoposide-treated cells (Fig. 6b and c). TPCK did not induce internucleosomal cleavage of DNA, a classical hallmark of apoptosis. Rather, it inhibited internucleosomal cleavage of DNA induced by many other apoptotic stimuli including etoposide in several different cell systems (Ghibelli et al., 1995; Yoshida et al., 1996; Zhu et al., 1997). This suggested that at least two different endonucleases were involved in the cleavage of DNA, one responsible for the formation of large

fragments of 200-300 and 30-50 kilobase pairs, and the other for the internucleosomal fragments, and that a TPCK inhibitable protease(s) may be closely associated with the latter endonuclease. The relationship between partial chromatin condensation and lack of internucleosomal cleavage of DNA during TPCK-induced apoptosis in THP.1 cells has not been investigated, nevertheless these two events were shown to be correlated in thymocyte apoptosis (Brown et al., 1993; G. Cohen et al., 1992; Fearnhead et al., 1995b). Furthermore, the differences in Ac-DEVD.AFC cleavage activity in lysates from TPCK- and etoposide-treated cells (Fig. 9) suggested that TPCK itself may have some inhibitory effect on activities of caspases after their activation. Indeed, TPCK (100 μM) has been shown to inhibit the ability of bacterially-expressed recombinant caspases-3 and -7 to cleave PARP (Fernandes-Alnemri et al., 1995b). Whether the concentration of TPCK required to exhibit similar effect could be achieved within intact cells remains to be determined. More importantly, the difference in the formation of the large subunits of caspase-3 (Fig. 10A) suggested that different mechanisms of caspase activation may be involved in TPCK- and etoposide-induced apoptosis. It has been shown that cells treated with TPCK, at the concentration which induced apoptosis, exhibited decreased trypsin-like serine protease activity as measured by Boc-Val-Leu-Lys.AMC cleavage activity (Zhu et al., 1997). This may represent one of the TPCK targets, possibly a serine protease(s), whose inhibition results in apoptosis. The protease responsible for this cleavage activity is, however, unknown, and thus the mechanism whereby TPCK induces apoptosis is yet to be elucidated.

3.6.2 Mechanisms of apoptosis induced by etoposide

The mechanisms of apoptosis induced by etoposide may be slightly better understood. Etoposide is a chemotherapeutic drug used in the treatment of several types of cancer. The cytotoxicity of the drug mainly results from its interaction with topoisomerase II, which both enhances the enzyme-mediated DNA breakage and inhibits the ability of the enzyme to religate cleaved DNA (Froelich-Ammon and Osheroff, 1995; Burden et al., 1996), resulting in the formation of permanent breaks. The presence of these breaks stimulates DNA recombination/mutation (Han et al., 1993), formation of chromosomal abnormalities (Lonn et al., 1989) and eventually triggers cell death by apoptosis (Kaufmann, 1989; reviewed by Hickman, 1992). Among the proposed

mechanisms of apoptosis induced by DNA-damaging agents, such as etoposide, are p53-mediated pathways. The tumour suppressor protein p53 is believed to become activated as a transcription factor in response to DNA damage and this activation may require DNA-dependent protein kinase activity (Woo et al., 1998). Activated p53 then induces transcriptional activation of a number of genes (reviewed by Levine, 1997). One of the genes directly activated by p53 includes *bax* (Miyashita and Reed, 1995), whose protein product can bind to Bcl-2 and prevent its ability to block apoptosis (Oltvai et al., 1993). Bax has also been shown to suppress tumour growth by stimulating apoptosis *in vivo* in a p53-dependent manner (Yin et al., 1997). Thus, transcriptional activation of *bax* gene by p53 can induce apoptosis. Another pathway proposed recently involves reactive oxygen species (ROS) (Polyak et al., 1997). Upon activation, p53 transcriptionally activates a specific subset of redox-related genes, resulting in the production of reactive oxygen species (ROS), which in turn damage mitochondria. Leakage of proteinaceous components from damaged mitochondria may then activate caspases during the apoptotic process (Kroemer et al., 1997). p53 has also been shown to have a transcription-independent function in apoptosis, as p53-dependent apoptosis proceeded in the absence of novel mRNA or protein synthesis (Caelles et al., 1994). In addition, mutant p53 protein could induce apoptosis without activating promoters of target genes (Haupt et al., 1995; Chen et al., 1996). Thus, it seems likely that in p53-mediated apoptosis there exists transcription-dependent and -independent pathways and that depending on cell types and stimulus, either one pathway or a combination of the two may be involved in committing cells to undergo apoptosis. As THP.1 cells were shown to express mutant p53 protein (Sugimoto et al., 1992), it is possibe that apoptosis induced by etoposide may be mediated by a p53 transcription-independent mechanism. However, the observation that mitogenically activated T lymphocytes and proliferating lymphoma cells from p53-null mice can undergo apoptosis after irradiation (Strasser et al., 1994) suggested the existence of a p53-independent mechanism of apoptosis in response to DNA damage. Etoposide was also shown to induce apoptosis in immature thymocytes obtained from p53-null mice (MacFarlane et al., 1996). Thus it is conceivable that p53-independent pathways also operate in DNA damage-induced apoptosis.

Recently, it has been shown that in p53-mediated apoptosis, caspase activation is required following UV irradiation (Fuchs et al., 1997) or etoposide treatment (Benjamin et al., 1998). The results described earlier (Fig. 10), together with the previous study (MacFarlane et al., 1997), also demonstrate that caspases are involved in etoposide-induced apoptosis in THP.1 cells. Moreover, caspases are also activated in etoposide-induced apoptosis in a p53 null cell line, HL-60 (Martins et al., 1997). These observations suggested that DNA damage by diverse stimuli can lead to caspase activation through p53-dependent and -independent pathways, thus supporting the hypothesis that multiple sensors of celluar insults converge on the central apoptotic machinery, the caspases (Cohen, 1997). It is therefore imperative to understand the biochemical pathways leading to caspase activation during the induction of apoptosis in order to gain further insight into the mechanisms of the cytotoxicity of DNA damaging agents.

Chapter 4

Dissociation of plasma membrane changes in cells undergoing apoptosis from other features of the apoptotic program

4.1 Introduction

In the previous chapter, apoptosis induced by TPCK or etoposide was characterised by morphological and biochemical changes affecting the nucleus, cytoplasm and plasma membrane. These changes in various cellular compartments are widely regarded as mechanistically linked events in a single "program", in which activation of caspases and proteolysis of intracellular substrates represent a final common pathway leading to cell death. There has been very limited exploration of the linkage of this program to the plasma membrane changes, which bring about swift recognition, phagocytosis, and safe degradation of apoptotic cells. *In vivo* intact cells dying by apoptosis are usually swiftly recognised and safely cleared by phagocytes (Duvall et al., 1985; Savill et al., 1993). This protects surrounding tissues from exposure to injurious contents leaking from dying cells, which is inevitable in accidental cell death by necrosis. Although there is only limited understanding of the molecular mechanisms which render apoptotic cells recognisable to phagocytes (Savill, 1998), exposure of phosphatidylserine (PS) appears to serve as a recognition signal for yet to be characterised PS receptors on certain phagocyte populations (Fadok et al., 1992a and b; Martin et al., 1995b; Verhoven et al., 1995). The mechanisms mediating PS exposure are complex but seem to involve activation of caspases (Vanags et al., 1996; Martin et al., 1996). Another mechanism by which phagocytes recognise cells undergoing apoptosis involves the phagocyte adhesion receptor CD36 co-operating with the $\alpha_v\beta_3$ vitronectin receptor integrin to bind thrombospondin (TSP), which acts as a molecular bridge between the apoptotic cell and the macrophage (Savill et al., 1990; Fadok et al., 1992a).

Based on the results described in the previous chapter, the apoptotic changes affecting the nucleus, cytoplasm and plasma membrane all appear to occur simultaneously making it difficult to establish the temporal relationships between these events including, particularly, the activation of caspases, reduction in mitochondrial membrane potential ($\Delta\Psi_m$) and PS exposure. To test the hypothesis that these changes are mechanistically linked events in a single "program" of apoptosis, a caspase inhibitor was used. This tripeptide, benzyloxycarbonyl-valinyl-alanyl-aspartyl (OMe) fluoromethyl ketone (Z-VAD.fmk), is a cell-permeable irreversible inhibitor of caspases whose permeability is facilitated by the presence of

benzyloxycarbonyl and OMe groups (Cohen, 1997). It inhibited apoptosis induced by a variety of stimuli in a number of cell systems, including rat thymocytes (Fearnhead et al., 1995a) and hepatocytes (Cain et al., 1996), human leukaemic Jurkat T cells (Chow et al., 1995; Armstrong et al., 1996), human B-cell lymphoma cell line GM701 and fibroblast cell line SKW6.4 (Jacobson et al., 1996), as well as monocytic THP.1 cells (Zhu et al., 1995). Moreover, in order to investigate the possible relationship between reduction in mitochondrial $\Delta\Psi_m$ and other features of apoptosis such as PS exposure, two mitochondrial inhibitors antimycin A and oligomycin were also used in the study. Antimycin A and oligomycin are inhibitors of the mitochondrial respiratory chain and ATP synthase respectively (Nicholls and Ferguson, 1992). Both induce apoptosis in several human lymphoblastoid cell lines (Wolvetang et al., 1994), but also inhibit thymocyte apoptosis induced by dexamethasone (Stefanelli et al., 1997). Nevertheless, these two inhibitors have often been used to modulate the changes in $\Delta\Psi_m$ in studies of apoptosis (Zamzami et al., 1995; Castedo et al., 1996).

4.2 Activation of caspases occurred upstream of both reduction in $\Delta\Psi_m$ and externalisation of PS, and also prior to increased Hoechst 33342 staining

It has been shown that treatment of THP.1 cells with etoposide, or TPCK, resulted in apoptosis, as assessed flow cytometrically by an increase in Hoechst 33342 fluorescence (Zhu et al., 1995 and 1997; MacFarlane et al., 1997). The increased Hoechst 33342 fluorescence is due to an increase in cell membrane permeability of apoptotic cells compared with control cells (Ormerod et al., 1993). In the present study, apoptosis was assessed by three independent flow cytometric methods measuring increase in Hoechst 33342 fluorescence, externalisation of PS, and a decrease in $\Delta\Psi_m$, respectively. Incubation of THP.1 cells for 4 h with either etoposide or TPCK induced apoptosis, as assessed by all three flow cytometric methods (Fig. 11A and B). Both antimycin A and oligomycin caused a concentration-dependent inhibition of apoptosis induced by the two stimuli, as assessed either by PS exposure or Hoechst 33342 staining whereas they had little effect on the decreased $\Delta\Psi_m$ (Fig. 11). Neither antimycin A nor oligomycin alone induced apoptosis as assessed by PS exposure, Hoechst 33342 staining or DNA fragmentation (data not shown). Thus

these experiments dissociated those features of the apoptotic phenotype assessed by PS exposure and Hoechst 33342 staining from those measured by the changes in $\Delta\Psi_m$.

Z-VAD.FMK (50 μM), a broad spectrum inhibitor of caspases, inhibited apoptosis induced by both etoposide and TPCK, as assessed by all three methods (Fig.11), providing further evidence of the involvement of caspases in the execution phase of apoptosis in THP.1 cells. More importantly, these results, together with the observation made in the time-course study described in the previous chapter, demonstrated that in this model of apoptosis the activation of caspases occurred upstream of both reduction in $\Delta\Psi_m$ and externalisation of PS, and also prior to increased Hoechst 33342 staining.

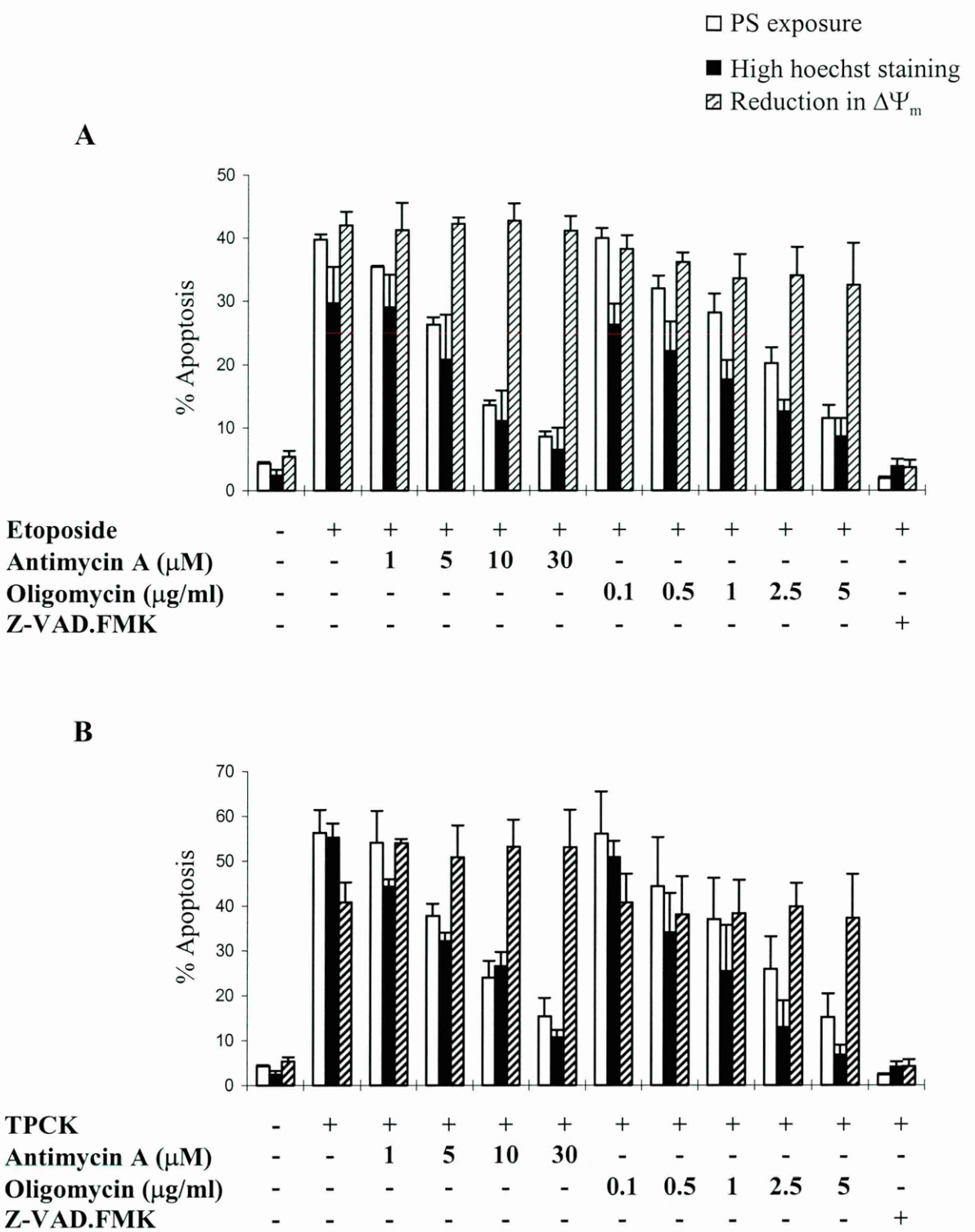


Figure 11. Antimycin A and oligomycin cause a concentration-dependent inhibition of externalisation of PS and increase in Hoechst 33342 fluorescence but not of the reduction in $\Delta\Psi_m$

THP.1 cells were incubated for 4 h with either (A) etoposide (25 μM) or (B) TPCK (75 μM) either alone or in the presence of the indicated concentrations of antimycin A (1 - 30 μM) or oligomycin (0.1 - 5 μg/ml) or in the presence of Z-VAD.FMK (50 μM). The percentage of apoptotic cells was then measured flow cytometrically using either increase in externalisation of PS, or increase in Hoechst 33342 fluorescence or decrease in $\Delta\Psi_m$. The data represent the mean (± sem) of at least three experiments.

4.3 Dissociation of PS externalisation from activation of caspases-3 and -7, and cleavage of PARP

During the execution phase of apoptosis, caspases are activated and subsequently cleave cellular substrates, such as PARP (Fig. 10 in chapter 3). Questions were therefore asked as whether the mitochondrial inhibitors differentially affected the activation of caspases and the cleavage of PARP. Using Western blot analysis, control cells were shown to contain the intact form of PARP (116 kDa) (Fig. 12A lane 1). Induction of apoptosis by either TPCK or etoposide was accompanied by the cleavage of PARP to its 85 kDa signature fragment (Fig. 12A lanes 2 and 6), which was completely blocked by Z-VAD.FMK (50 μM) (Fig. 12A lanes 5 and 9). Neither oligomycin nor antimycin A blocked this proteolysis of PARP following incubation with either TPCK (Fig. 12A lanes 3 and 4) or etoposide (Fig. 12A lanes 7 and 8). The cleavage of PARP to its 85 kDa signature fragment, which occurs at a DEVD↓G sequence separating the amino terminal DNA binding domain and carboxy terminal catalytic domain of the enzyme (Lazebnik et al., 1994), suggested the activation of caspase-3 and/or -7, which were examined using antibodies raised against their respective large subunits. Control THP.1 cells showed the presence of the intact proform of caspase-3 (Fig. 12B lane 1). Induction of apoptosis with either TPCK or etoposide was accompanied by loss of the proform of caspase-3 together with formation of the catalytically active large subunit (LS) of caspase-3 (Fig. 12B lanes 2 and 6). Cleavage of caspase-3 was more extensive in response to TPCK compared with etoposide, commensurate with the greater induction of apoptosis by this stimulus (Fig. 11). Z-VAD.FMK, which blocked the induction of apoptosis induced by these stimuli (Fig. 11), almost totally blocked the formation of the large subunit of caspase-3 in cells treated with either TPCK (Fig. 12B lane 5) or etoposide (Fig. 12B lane 9), although a small amount of a slightly larger immunoreactive fragment (~ p20) was also observed (Fig. 12B lane 9). This fragment was most probably catalytically inactive as no cleavage of PARP was detected (Fig. 11A lane 9). Treatment with TPCK or etoposide also resulted in the activation of caspase-7 with the formation of a catalytically active large subunit (LS) (Fig. 12C lanes 2 and 6), whereas untreated control cells retained the intact proform of caspase-7 (Fig. 12C lane 1). Again, Z-VAD.FMK completely blocked the formation of the large subunit of caspase-7 in cells treated with either TPCK (Fig. 12C lane 5) or etoposide (Fig. 12C lane 9). Thus,

these results clearly demonstrated that Z-VAD.FMK inhibited apoptosis by blocking the processing/activation of caspases, in agreement with the previous observations (Slee et al., 1996; MacFarlane et al., 1997; Zhu et al., 1997). In contrast, neither oligomycin nor antimycin A inhibited the formation of respective large subunits (LS) of caspases-3 and -7 after treatment with either TPCK (Fig. 12B and C lanes 3 and 4) or etoposide (Fig. 12B and C lanes 7 and 8). Thus oligomycin and antimycin A inhibited the externalisation of PS and the increase in cell membrane permeability assessed by Hoechst 33342 staining (Fig. 11) but did not inhibit the activation of caspases-3 and -7, and cleavage of PARP (Fig. 12).

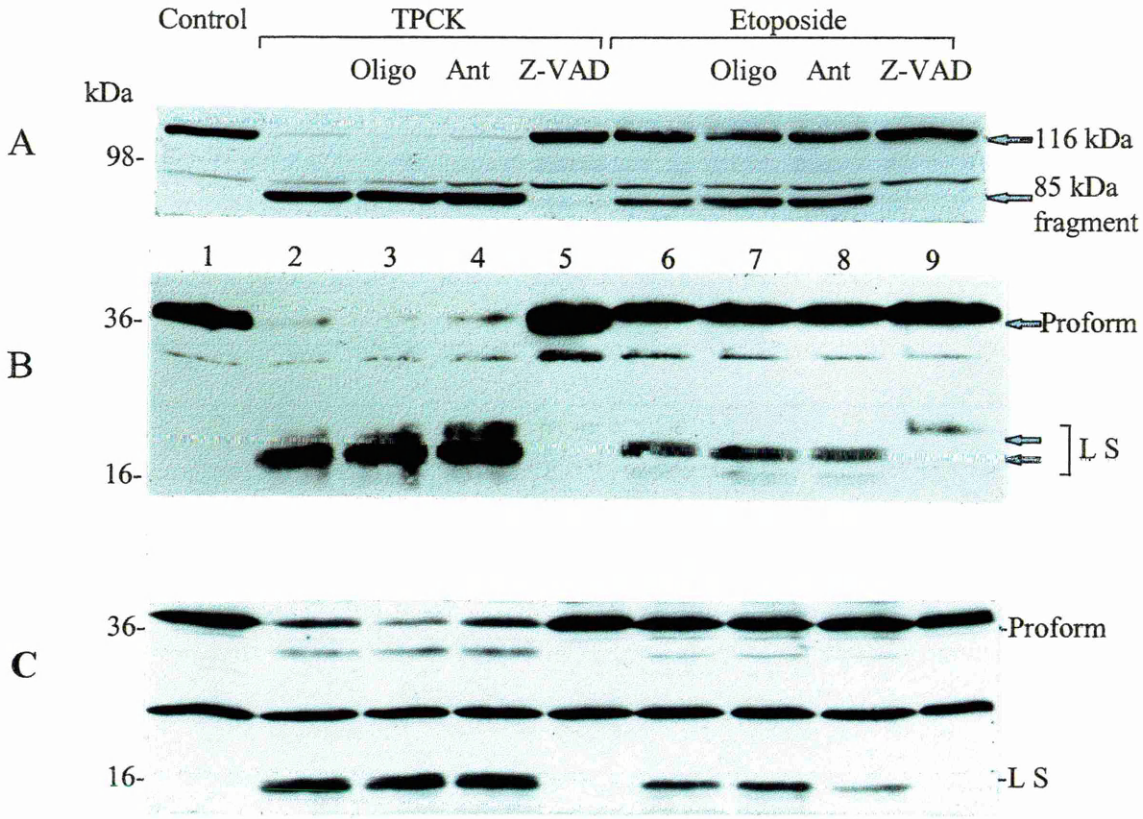


Figure 12. Z-VAD.FMK but not antimycin A or oligomycin inhibits PARP cleavage and activation of caspases-3 and -7 in THP.1 cells.

THP.1 cells were incubated for 4 h with either TPCK (75 μM) or etoposide (25 μM) in the presence or absence of oligomycin (Oligo 5 μg/ml), antimycin A (Ant 30 μM) or Z-VAD.FMK (Z-VAD 50 μM). To detect the 85 kDa fragment of PARP (A), cellular proteins were resolved on an SDS-7% polyacrylamide gel, transferred onto nitrocellulose membrane and probed with rabbit antiserum (318). To detect activation of caspases-3 (B) and -7(C), cellular proteins were resolved on an SDS-15% polyacrylamide gel. After transferring, the proteins were probed with rabbit polyclonal antibodies raised against the large subunits (LS) of caspase-3 and -7, respectively. Results are representative of three experiments.

4.4 Dissociation of PS externalisation from DNA fragmentation

To further determine whether the treatment with antimycin A or oligomycin had interfered with the DNA fragmentation which occurs late in apoptosis, conventional agarose gel electrophoresis was carried out to examine the effect of the two inhibitors on internucleosomal cleavage of DNA. Etoposide alone induced internucleosomal DNA cleavage compared with control cells (Fig. 13 compare lanes 1 and 2). Z-VAD.FMK but not antimycin A or oligomycin inhibited etoposide-induced internucleosomal cleavage (Fig. 13 lanes 3-5). TPCK alone induced fragmentation of DNA to large fragments of 200-300 and 600-700 kilobase pairs in length in the absence of internucleosomal cleavage (Fig. 7 in chapter 3, and Zhu et al., 1997). Z-VAD.FMK but not antimcycin A or oligomycin inhibited the formation of these large DNA fragments (data not shown). Thus externalisation of PS during apoptosis can also be dissociated from DNA fragmentation.

4.5 Cell surface changes resulting in phagocytic recognition can be dissociated from other features of the apoptotic phenotype

One of the key features of the apoptotic process is the rapid recognition and subsequent phagocytosis of apoptotic cells. As both antimycin A and oligomycin inhibited plasma membrane changes, such as externalisation of PS and increased Hoechst staining, but not other features of the apoptotic phenotype, the question arose as to whether they also interfered with phagocytic recognition of apoptotic THP.1 cells. Peritoneal and bone marrow macrophages phagocytose apoptotic cells utilising receptors for PS (PSRs) or thrombospondin (i.e. $\alpha_v\beta_3$ and CD36) respectively (Savill et al., 1990 and 1993; Fadok et al., 1992a and b). Exposure of THP.1 cells to the two pro-apoptotic stimuli, etoposide and TPCK, resulted in an increase in the percentage of cells phagocytosed by both peritoneal macrophages and bone marrow-derived macrophages (Table 3). Z-VAD.FMK, antimycin A and oligomycin, all inhibited the etoposide- and TPCK-induced increases in recognition and phagocytosis by both peritoneal and bone marrow-derived macrophages (Table 3). Thus cells displaying many features of apoptosis including reduction in $\Delta\psi_m$, DNA fragmentation, processing of caspases-3 and -7, and PARP cleavage do not display characteristic cell

surface changes, such as PS externalisation, which result in phagocytic recognition and engulfment.

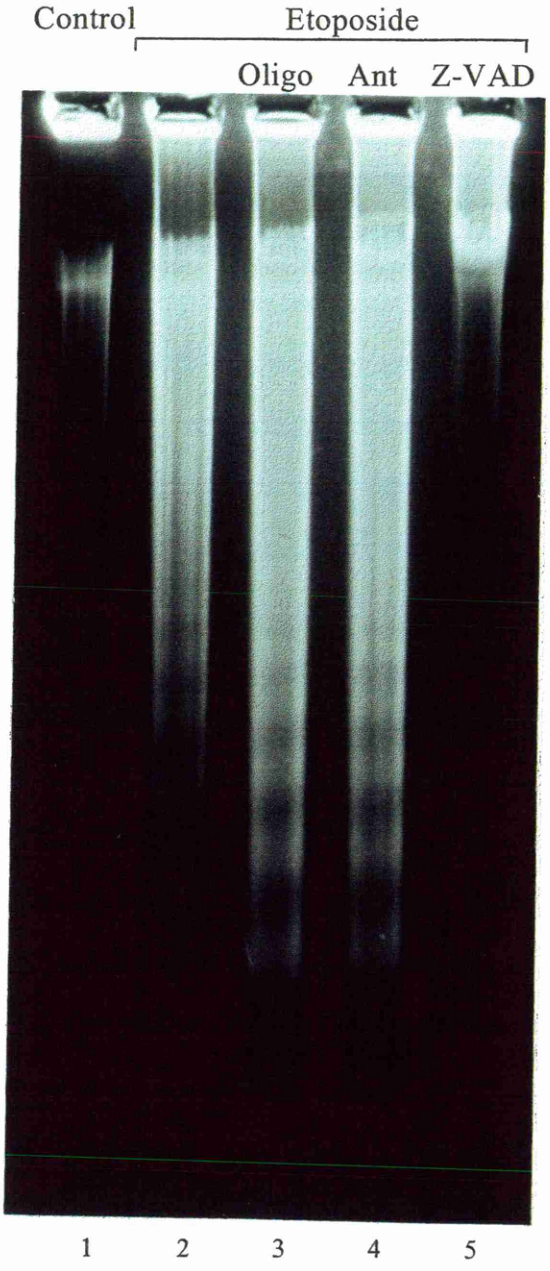


Figure 13. Neither antimcycin A nor oligomycin inhibits internucleosomal cleavage of DNA in THP.1 cells.

Induction of apoptosis in THP.1 cells was essentially as described in the legend to Fig. 2. 2 x 10^6 cells were loaded per lane and subjected to conventional agarose gel electrophoresis to detect internucleosomal cleavage of DNA. Results are representative of three experiments.

Table 3. Inhibition of phagocytosis of apoptotic THP.1 cells by oligomycin and antimycin A*

Treatment	% Phagocytosis	
	Peritoneal macrophages	Bone-marrow macrophages
Control	3.1 ± 0.9	1.7 ± 0.5
TPCK	25.6 ± 5.3	19.7 ± 2.8
TPCK + Z-VAD.FMK	4.4 ± 1.1	3.8 ± 1.1
TPCK + Oligomycin	4.9 ± 1.6	1.1 ± 0.5
TPCK + Antimycin A	4.5 ± 0.5	2.4 ± 0.8
Etoposide	13.0 ± 2.3	10.7 ± 0.6
Etoposide + Z-VAD.FMK	5.2 ± 3.0	2.2 ± 0.3
Etoposide + Oligomycin	3.9 ± 1.6	1.4 ± 0.5
Etoposide + Antimycin A	3.8 ± 0.5	1.0 ± 0.1

Induction of apoptosis in THP.1 cells was essentially as described in the legend to Fig. 12. Phagocytosis was evaluated by counting 500 macrophages per slide of four replicate slides in each experiment (see Materials and methods). Peritoneal and bone-marrow macrophages phagocytose apoptotic cells utilising receptors for PS and thrombospondin respectively. Results were expressed as a percentage of macrophages that had phagocytosed THP.1 cells. The data represent the mean (± sem) of three experiments.

* This work was made possible essentially through the collaboration with Dr. J. Savill and his colleagues at the University of Nottingham.

4.6 Dissociation of cell membrane changes from caspase activation during induction of apoptosis in U937 cells

In order to determine whether the phenomenon of dissociation of externalisation of PS from other features of the apoptotic phenotype was cell type-specific, the study was extended to human leukaemic U937 cells. These cells were treated with two commonly used apoptotic stimuli, etoposide (25 μM) or tumour necrosis factor-α (TNF-α, 10 ng/ml) in the presence of cycloheximide (0.9 μM) (Vanags et al., 1996). Apoptosis was induced by both these stimuli, as assessed by externalisation of PS, increase in Hoechst 33342 fluorescence and increase in cells with a decreased $\Delta\Psi_m$ (Fig. 14A). Both oligomycin (5 μg/ml) and antimycin A (30 μM) largely blocked the apoptotic changes induced by either TNF-α or etoposide as assessed by either PS exposure or Hoechst 33342 staining but had little effect on the decreased $\Delta\Psi_m$ (Fig. 14A). Z-VAD.FMK (20 μM) inhibited all the apoptotic changes induced by etoposide whereas at a concentration of 2 μM it effectively inhibited TNF-α-induced apoptosis, as assessed by all three methods (Fig. 14A). It was observed that Z-VAD.FMK at the concentrations higher than 2 μM potentiated necrosis in cells treated with TNF-α in the presence of cycloheximide (data not shown). Western blot analysis showed that control U937 cells contained almost entirely intact PARP (116 kDa) (Fig. 14B lane 1). Induction of apoptosis by either TNF-α or etoposide was accompanied by activation of caspases as assessed by the cleavage of PARP to its 85 kDa signature fragment (Fig. 14B lanes 2 and 6). This cleavage was completely blocked by Z-VAD.FMK (Fig. 14B lanes 5 and 9), providing further evidence for the activation of caspases during apoptosis. Apoptosis, as assessed by PS exposure, Hoechst 33342 staining or PARP cleavage was not induced by either antimycin A or oligomycin alone (data not shown). Neither antimycin A nor oligomycin blocked proteolysis of PARP to its 85 kDa fragment following co-incubation with either TNF-α (Fig. 14B lanes 3 and 4) or etoposide (Fig. 14B lanes 7 and 8). Thus, these results in U937 cells dissociated those features of the apoptotic phenotype assessed by PS exposure and Hoechst 33342 staining from those measured by the changes in $\Delta\Psi_m$ and caspase activation.

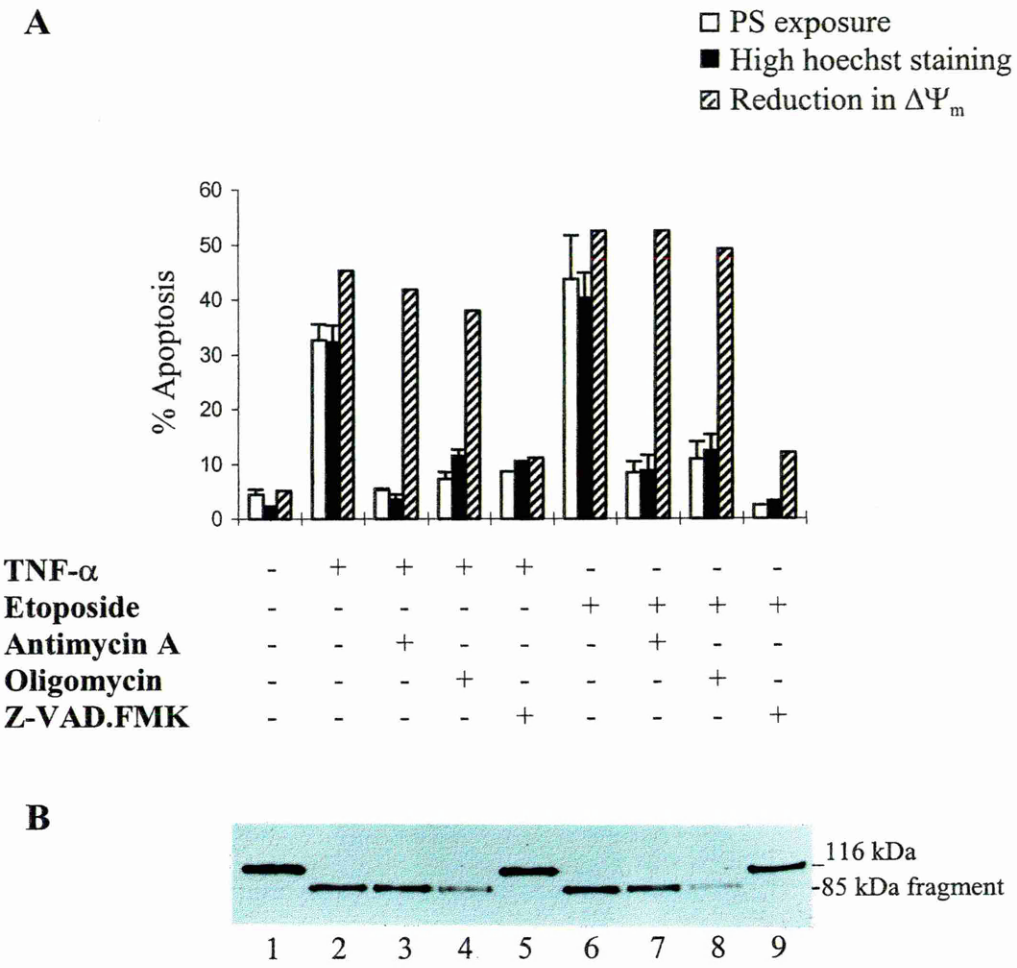


Figure 14. Dissociation of PS externalisation from caspase activation in apoptotic U937 cells.

Apoptosis was induced in U937 cells following incubation for either 3 h with tumour necrosis factor-α (TNF-α) (10 ng/ml) in the presence of cycloheximide (0.9 μM) (lanes 2-5) or 5 h with etoposide (25 μM) (lanes 6-9). U937 cells were incubated either alone (lane 1) or with the apoptotic stimuli in the presence of antimycin A (30 μM) (lanes 3 and 7) or oligomycin (5 μg/ml) (lanes 4 and 8). Cells were also incubated with the apoptotic stimuli in the presence of the caspase inhibitor Z-VAD.FMK, the concentration of which was either 2 or 20 μM in the case of TNF-α or etoposide respectively (lanes 5 and 9). (A) The percentage of apoptotic cells was then measured as described in legend to Fig. 1. The data represent the mean (± sem) of at least three experiments except for $\Delta\Psi_m$, which represents the mean of two determinations. (B) Cellular proteins were resolved on SDS-10 % polyacrylamide gels, transferred onto nitrocellulose membrane and probed with a mouse monoclonal antibody (C2-10) to detect both intact PARP (116 kDa) and its cleaved 85 kDa fragment.

4.7 Discussion

4.7.1 Caspases act upstream of PS externalisation and decrease in $\Delta\Psi_m$

Apoptosis induced in both THP.1 and U937 cells was assessed by three independent flow cytometric methods measuring an increase in PS exposure, increased Hoechst 33342 fluorescence and an increase in the number of cells with decreased $\Delta\Psi_m$ (Figs. 11 and 14A). The induction of apoptosis in both cell lines was also accompanied by the activation of caspases with subsequent cleavage of PARP (Figs. 12 and 14B). Z-VAD.FMK inhibited activation of caspases-3 and -7 during the induction of apoptosis in THP.1 cells and activities of caspases responsible for PARP cleavage in both THP.1 and U937 cells. This cell membrane permeable inhibitor of caspases also inhibited all the biochemical changes associated with apoptosis, including PS exposure, increased Hoechst 33342 binding, and decreased $\Delta\Psi_m$. These results clearly demonstrate that caspase activation occurs upstream of reduction in $\Delta\Psi_m$, and also prior to externalisation of PS. This observation is compatible with some recent studies (Yang et al., 1997; Kluck et al.,1997; Bossy-Wetzel et al.,1998), but in disagreement with others (Shimizu et al., 1996; Hirsch et al., 1997). The discrepancy could be due to several reasons. In the study reported by Shimizu et al. (1996), it was shown that rat pheochromocytoma PC12 cells transfected with Bcl-2 and Bcl-x_L maintained normal mitochondrial $\Delta\Psi_m$ during the induction of apoptosis by proapototic stimuli including etoposide and became resistant to cell death. Cells co-incubated with the caspase inhibitor Ac-YVAD.CHO showed a rapid decrease in $\Delta\Psi_m$ and only partial protection against apoptosis, concluding that caspases functions downstream of reduction in $\Delta\Psi_m$. However, previous work in this laboratory has already shown that Ac-YVAD.CHO, a specific inhibitor of caspase-1 (Thornberry et al., 1992), was incapable of blocking processing of caspases-2 and -3, and thus did not inhibit apoptosis induced by cycloheximide in the presence of trysin-like serine protease inhibitor TLCK in THP.1 cells, although it inhibited lamin A/B cleavage (MacFarlane et al., 1997). Thus different caspase inhibitors used in the two studies may account for the observed difference. In another study, it was concluded that caspases were activated after the development of mitochondrial permeability transition (PT) as indicated by a decreased $\Delta\Psi_m$ (Hirsch et al. 1997). This conclusion was based essentially on experiments of modulating PT by using some pharmacological agents, in which PT inducers alone caused the apoptosis and PT

inhibitors blocked thymocytes apoptosis induced by dexamethasone or etoposide, and in which Z-VAD.FMK did not prevent the disruption of $\Delta\Psi_m$ induced by the PT inducers. Obviously, using pharmacological agents to modulate PT is a useful approach to study the role of individual biochemical events in the pathways leading to activation of the execution machinery in apoptosis. However, whether and how much this approach represents the physiological situation within an intact cell are not clear. Added to this, the specificities of modes of action by the pharmacological agents are always subject to dispute. Thus particular caution may have to be exercised in the interpretation of these results.

4.7.2 Dissociation of phagocytic recognition of cells undergoing apoptosis from other features of the apoptotic program

The data presented here also demonstrate the dissociation of phagocyte recognition of cells undergoing apoptosis from other features of this programmed form of cell death. The mitochondrial inhibitors, antimycin A and oligomycin, not only inhibited surface changes of apoptosis, such as externalisation of PS recognised by murine peritoneal macrophages but also inhibited surface changes recognised by murine bone marrow-derived macrophages, which use the CD36/$\alpha_v\beta_3$ and thrombospondin recognition mechanism (Tables 3). However, antimycin A and oligomycin did not inhibit activation of caspases-3 and -7, cleavage of PARP and DNA degradation (Figs. 12 and 13), and the reduction in mitochondrial $\Delta\Psi_m$ (Fig. 11). These data imply that the surface changes of apoptosis relate to an independent pathway of events, which may be differentially regulated from the activation of "effector" caspases which are believed to trigger the nuclear and some of the cytoplasmic changes of apoptosis. It has become a widely held view that PS exposure and associated changes, such as increased plasma membrane permeability to Hoechst dye, represent "early" features of a single, co-ordinately regulated common pathway leading to cell death, while DNA degradation is a "late" feature. However, temporal dissociation of membrane changes including PS exposure from other features of apoptosis could also imply that mechanisms regulating the ability of dying cells to be recognised by phagocytes are part of a parallel process, which may be independent from other features of the program. This interpretation of the data is strongly supported by the effects of antimycin A and oligomycin reported here. Future studies will need to characterise

the pathway by which dying cells display "eat me" signals. The effects of Z-VAD.FMK reported here support previous data implicating the activation of some caspases in the externalisation of PS (Vanags et al., 1996; Martin et al., 1996), although "effector" caspases such as caspases-3 and -7 appear not to be involved.

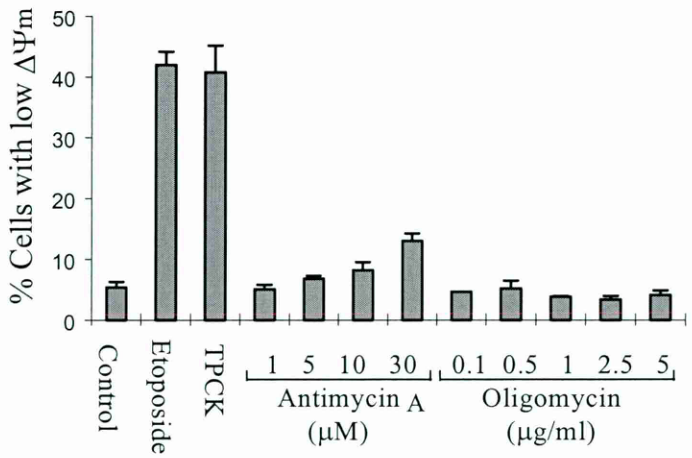
In the present study, PS exposure was also uncoupled from the reduction in $\Delta\Psi_m$. There was excellent agreement between the two methods detecting respective increases in Hoechst 33342 fluorescence and externalisation of PS, suggesting that they may be measuring very similar characteristics of the apoptotic cells. While values for the decreased $\Delta\Psi_m$ were generally in reasonable agreement with the other measures of apoptosis, there were notable exception in particular with antimycin A and oligomycin, where the two inhibitors dissociated those features of the apoptotic phenotype assessed by PS exposure and increased Hoechst staining from those measured by the changes in $\Delta\Psi_m$. This suggested that the decreased $\Delta\Psi_m$ was measuring a different characteristic of apoptosis compared with those measured by externalisation of PS and increased Hoechst staining. The dissociation of apoptotic features described here also provides a cautious note on the methods of assessing apoptosis. Externalisation of PS is a commonly used marker of apoptosis. The results from this study demonstrated that to assess apoptosis it is preferable to utilise several methods, which reflect different characteristics of the apoptotic phenotype, rather than using any single method.

4.7.3 Mechanisms of the dissociation of apoptotic features

Although it is possible to dissociate those cell surface changes leading to phagocytic recognition and engulfment from other cytoplasmic, mitochondrial and nuclear changes of apoptosis, the mechanisms of this dissociation have yet to be understood. Under the present experimental conditions, neither antimycin A nor oligomycin alone, at the concentrations which inhibit cell surface changes including PS exposure, induced a significant reduction in mitochondrial membrane potential ($\Delta\psi_m$) (Fig. 15A). Furthermore, both mitochondrial inhibitors alone did not induce a loss of ATP in THP.1 cells (data not shown) nor did they potentiate the decrease in ATP levels during the induction of apoptosis by TPCK (Fig. 15B). These observations raised the possibility that the two inhibitors may not be exerting their effects primarily on

mitochondria to bring about the inhibition of cell surface changes of the apoptotic cells. However, it has been reported that the inhibition of electron transport by antimycin A or of ATP synthesis by oligomycin alone may not depolarise the mitochondria completely provided that a supply of ATP is available through glycolysis (Mohr and Fewtrell, 1990; Rizzuto et al., 1994; Budd and Nicholls, 1996b). It was proposed that mitochondria can maintain their membrane potential by running the ATP synthase "in reverse", i.e. by hydrolysing ATP to pump proton out of the mitochondrial matrix. Indeed, a recent study showed that several haematopoietic cell lines were insensitive to mitochondrial poisons and these cells generated ATP entirely by glycolysis, maintaining normal mitochondrial $\Delta\psi_m$ during the induction of apoptosis (Garland and Halestrap, 1997). Thus, the results of the present study are consistent with the above observations of others. In addition to the fundamental role as the cell's primary energy supplier, mitochondria have become increasingly well known in recent years as potent buffers of cytosolic Ca^{++} (Rizzuto et al., 1994; Hoth et al., 1997). The disturbances of mitochondrial function in intracellular Ca^{++} homeostasis by the two inhibitors at the concentrations used in the present study have been reported (Mohr and Fewtrell, 1990; Budd and Nicholls, 1996b; Hoth et al., 1997). Therefore the possibility that antimycin A or oligomycin may have modulated cytosolic Ca^{++} levels which subsequently resulted in the inhibition of cell surface changes on apoptotic cells can not be excluded. Neither antimycin A nor oligomycin alone directly blocked the binding of annexin V to PS residues on the outer plasma membrane of the apoptotic cells (data not shown). In agreement with a report that extracellular Ca^{++} is required for efficient PS exposure during apoptosis (Hampton et al., 1996), EGTA (2 mM) inhibited PS exposure, but not activation of caspases, during induction of apoptosis by either TPCK or etoposide in THP.1 cells (data not shown). However, it seems unlikely that antimycin A or oligomycin will have any effect on extracellular Ca^{++}.

A



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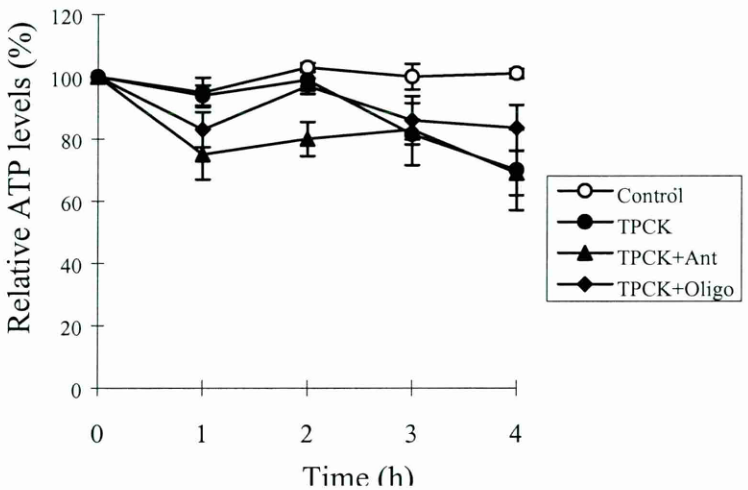


Figure 15. Both antimycin A and oligomycin alone did not induce a reduction in mitochondrial $\Delta\psi_m$ or potentiate a decrease in intracellular ATP levels during the induction of apoptosis.

(A) THP.1 cells were incubated for 4 h either alone or with etoposide (25 μM) or TPCK (75 μM). Cells were also incubated with the indicated concentrations of antimycin A (1-30 μM) or oligomycin (0.1-5 μg/ml) alone. The percentage of cells with decreased $\Delta\Psi_m$ was then determined flow cytometrically as described in Materials and Methods. (B) Cells were incubated for up to 4 h either alone or with TPCK (75 μM) in the presence or absence of antimycin A (30 μM) or oligomycin (5 μg/ml). At the indicated time points, relative cellular ATP levels (% of control cells at zero time point) were measured as described in Materials and Methods

It has been reported that PS exposure requires the activation of a Ca^{++}-dependent scramblase and/or the loss of activity of aminophospholipid translocase (Verhoven et al., 1995; Bratton et al., 1997). It is thus conceivable that the mitochondrial inhibitors may have suppressed the activity of the scramblase while stabilising the activity of the aminophospholipid translocase, so preventing the externalisation of PS. The preliminary results showed, however, that the two mitochondrial inhibitors did not appear to inhibit either the activation or the activity of the scramblase in human peripheral red blood cells (data not shown). Their effects on activity of aminophospholipid translocase have not yet been measured. Thus, future work is still required to elucidate the mechanisms of this dissociation.

Chapter 5

Release of mitochondrial cytochrome c occurs upstream of the activation of caspases

5.1 Introduction

The data presented in the previous chapters have clearly demonstrated that caspase activation occurs prior to the other major biochemical changes characteristic of the apoptotic phenotype, including the reduction in mitochondrial membrane potential ($\Delta\Psi_m$) during the induction of apoptosis by etoposide or TPCK in THP.1 cells. The mechanism initiating the activation of caspases in this model of apoptosis is, however, unclear. Recently, release of mitochondrial cytochrome c has been observed as an early feature in many models of apoptosis (Liu et al., 1996; Yang et al., 1997; Kluck et al., 1997). Further studies show that in the presence of dATP, cytochrome c interacts with apoptotic protease activating factor-1 (Apaf-1), resulting in the activation of caspase-9, which in turn activates other caspases, such as caspase-3 (Liu et al., 1996; P. Li et al., 1997; Zou et al., 1997). Several mechanisms for the release of mitochondrial cytochrome c are proposed, including a) opening of a mitochondrial permeability transition pore resulting in reduction in $\Delta\Psi_m$ and osmotic swelling, b) the presence of a specific channel for cytochrome c in the outer membrane or c) mitochondrial swelling and rupture of the outer membrane but without loss of mitochondrial $\Delta\Psi_m$ (reviewed by Reed, 1997b). However, these mechanisms do not appear to be generally applicable, as release of cytochrome c occurs in cells with normal mitochondrial membrane potential (Kluck et al., 1997; Yang et al., 1997; Bossy-Wetzel et al., 1998). Two recent studies have highlighted another possible mechanism for this release, involving Bid, a Bcl-2 interacting protein containing a proapoptotic BH3 domain (K. Wang et al., 1996). Cleavage of Bid by caspase-8 results in translocation of the cleaved Bid to the mitochondria where it induces the release of cytochrome c during cell surface death receptor-mediated apoptosis (Li et al., 1998; Luo et al., 1998). The BH3 domain of Bid is essential for its ability to induce the release of cytochrome c (Luo et al., 1998).

The primary aim of the present study is to investigate the importance of the release of mitochondrial cytochrome c in the induction of apoptosis by etoposide or TPCK. In addition, some of the proposed hypotheses for mechanisms of cytochrome c release were also investigated in this model of apoptosis in THP.1 cells.

5.2 Release of mitochondrial cytochrome c occurred upstream of the reduction in mitochondrial $\Delta\Psi_m$.

Incubation with etoposide resulted in a time-dependent induction of apoptosis, as assessed flow cytometrically by PS exposure, and decreased $\Delta\Psi_m$ (Fig. 16A). This treatment also resulted in a time-dependent cytosolic accumulation of cytochrome c, as detected by Western blot analysis (Fig. 16A lanes 2-6, *upper blot*). Significant changes in all these parameters were clearly observed after 2 h treatment. Z-VAD.FMK, the caspase inhibitor, completely blocked the etoposide-induced PS exposure. Although Z-VAD.FMK also clearly inhibited the fall in $\Delta\Psi_m$, its effect on cytochrome c release was remarkably different (Fig. 16A). Z-VAD.FMK did not block the etoposide-induced cytosolic accumulation of cytochrome c (Fig. 16A lane 7, *upper blot*). The absence of cytochrome oxidase (subunit II) in the cytosolic extracts confirmed that the samples were free from mitochondrial contamination (Fig. 16A, *lower blot*). Thus Z-VAD.FMK prevented all the biochemical changes of apoptosis including PS exposure, and the fall in $\Delta\Psi_m$ except for the release of mitochondrial cytochrome c. These data suggested that the release of cytochrome c occurred upstream or independent of the reduction in $\Delta\Psi_m$. Further support for this conclusion was provided by the data with TPCK, which caused a time-dependent increase in the percentage of cells with externalised PS and a reduced $\Delta\Psi_m$ (Fig. 16B lanes 2 - 6). This increase was prevented by Z-VAD.FMK (Fig. 16B lane 7). TPCK also induced a time-dependent release of mitochondrial cytochrome c (Fig. 16B lanes 2 - 6, *upper blot*), which was not blocked by Z-VAD.FMK (Fig. 16B lane 7). In TPCK-induced apoptosis the release of cytochrome c was already evident at 1h, earlier than either the externalisation of PS or the reduction in $\Delta\psi_m$ (Fig 16B). At 1 h, caspase-7 was also processed with the formation of catalytically-active large subunit, whereas cleavage of PARP was first observed at 2 h (see Figs. 10B and C in chapter 3). While these data did not distinguish whether cytochrome c release preceded the activation of caspase-7, results with both apoptotic stimuli demonstrated that the release of cytochrome c occurred upstream of the reduction in $\Delta\Psi_m$, during the induction of apoptosis.

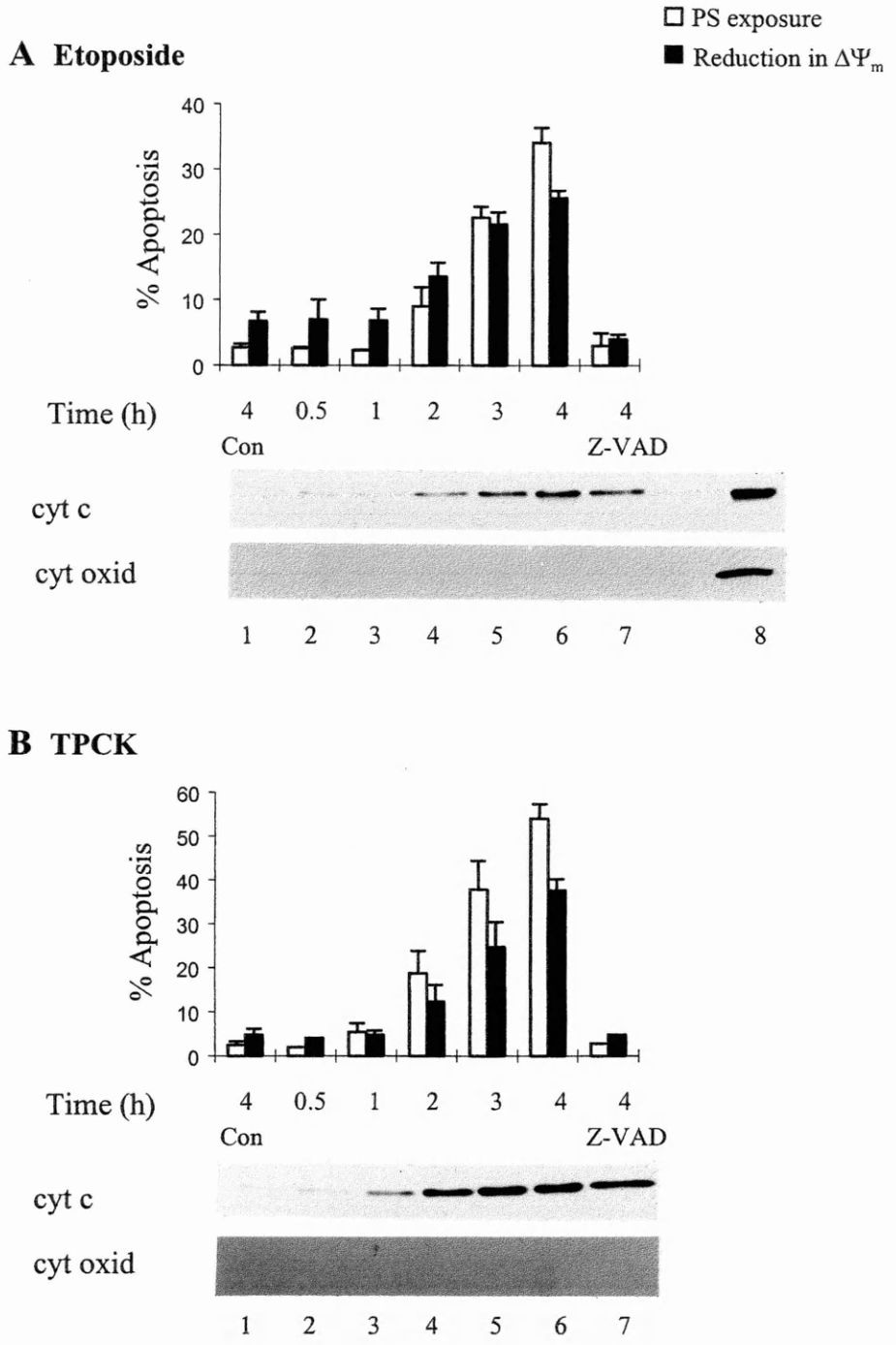


Figure 16. Cytochrome c release is upstream of the decrease in $\Delta\Psi_m$

(A) Time course of induction of apoptosis by etoposide (25 μM) was determined by PS exposure and decreased $\Delta\Psi_m$. Cells were incubated for the indicated times with etoposide (lanes 2 - 6) or in the presence of Z-VAD.FMK (50 μM) (lane 7). Cytosolic extracts were prepared and assessed by Western blot analysis for cytochrome c (cyt c) and also for cytochrome oxidase subunit II (cyt oxid), as a marker of mitochondrial contamination of the cytosolic extracts. Purified bovine heart cytochrome c (5 ng) (lane 8, upper blot) and 5 μl of the mitochondrial fraction following removal of the cytosolic extracts were loaded (lane 8, lower blot) as positive controls. (B) The time course of induction of apoptosis by TPCK (75 μM) (lanes 2 - 6) or in the presence of Z-VAD.FMK (50 μM) (lane 7) was determined as described above. Cytosolic extracts were analysed for cytochrome c and cytochrome oxidase II. Results were expressed as mean (± sem) of at least three experiments or representative of three experiments except for those in lanes 2 and 7 of (B), which represent mean of two experiments.

5.3 Mitochondrial ultracondensation and outer mitochondrial membrane discontinuities in apoptotic cells were inhibited by Z-VAD.FMK

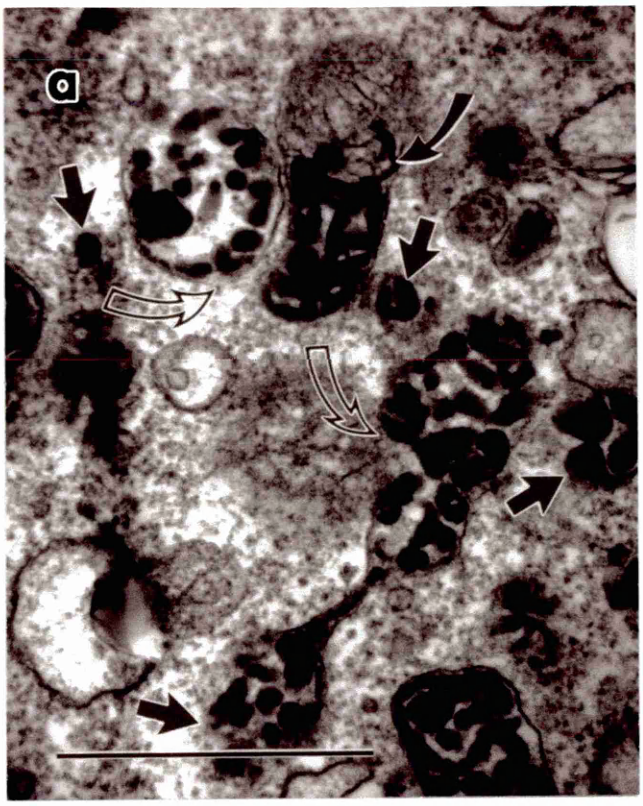
Many morphological changes of mitochondria including swelling and rupture of the outer membrane have been reported as critical events which result in the release of cytochrome c (Vander Heiden et al., 1997). Therefore the ultrastructure of mitochondria was examined during the induction of apoptosis. Unlike control cells, THP.1 cells incubated with etoposide developed many characteristic ultrastructural features of apoptosis. Cells treated with TPCK exhibited similar cytoplasmic changes but with less clumping and condensation of the heterochromatin (see Fig. 6 in chapter 3). Particularly striking was the presence of many ultracondensed mitochondria in both etoposide- and TPCK-treated cells (Fig. 17a and b). The time-course study revealed that a few of these ultracondensed mitochondria were observed as early as 2 h but were more prevalent by 4 h (data not shown). These mitochondria were defined as ultracondensed, based on their marked increase in matrix density and slight dilation of the outer compartment/intracristal space despite a marked reduction in volume. The reduced volume was inferred from their mean diameter (262 ± 58 nm) (mean ± sem) in sections, compared with that of controls (474 ± 109 nm). Similar ultracondensed mitochondria have been observed in tumour necrosis factor-induced apoptosis of a lymphoblastic cell line (Jia et al., 1997). These changes were distinct from the commonly described condensed mitochondria, which exhibit increased matrix density without any overall reduction in volume and correspond to state 3 respiration (Hackenbrock, 1968; Laiho and Trump, 1975). Ultracondensed mitochondria were invariably present in cells showing the nuclear and cytoplasmic changes but they were also found in some cells showing no other morphological signs of apoptosis (data not shown). While most mitochondria in the apoptotic cells were affected, some remained normal and a few showed condensation of only part of their inner compartment (Fig. 17a and b). Discontinuities were observed in the outer membrane profile of many of the ultracondensed mitochondria and, although some could be attributed to obliquity of the section, many indicated exposure of inner-membrane-limited regions of the condensed matrix to the cytosol (Fig. 17a and b). The contorted shapes of these organelles, together with the electron density of the surrounding cytoplasm in apoptotic cells militated against the clear identification of

discontinuities in ultrathin sections. True discontinuities were, however, recognised by tilting these sections during examination. Further evidence was obtained from 0.5-1.0 μm sections contrasted *en bloc* and examined using an integrated energy filter to remove inelastically scattered electrons from the image. Z-VAD.FMK inhibited all the ultrastructural changes induced by etoposide and TPCK, including the mitochondrial and nuclear condensation together with the development of outer mitochondrial membrane discontinuities (Figs. 17c and d). These results demonstrate that the formation of both ultracondensed mitochondria and discontinuities in the outer mitochondrial membrane occurred downstream of the activation of caspases.

Figure 17. Z-VAD.FMK inhibited the formation of mitochondrial ultracondensation and discontinuities during the induction of apoptosis in THP.1 cells.

(a) Cells incubated with etoposide (25 μM) for 4 h showed many ultracondensed mitochondria (solid arrows). The increase in matrix density was usually uniform but, in some mitochondria, only restricted regions were affected (solid curved arrow). (b) Similar changes were observed after treatment with TPCK (75 μM). Apparent discontinuities in the outer membrane of many of these mitochondria could be attributed to obliquity of the section (open curved arrows) but several of these organelles also showed disruption of this membrane resulting in the exposure of membrane-limited regions of the condensed matrix to the cytosol (solid arrows). (c) Co-incubation of THP.1 cells with etoposide and Z-VAD.FMK (50 μM) for 4 h resulted in complete inhibition of the formation of mitochondrial ultracondensation and discontinuities as well as other characteristic ultrastructural features of apoptosis. (d) Co-incubation of THP.1 cells with TPCK and Z-VAD.FMK (50 μM) for 4 h also prevented the formation of mitochondrial discontinuities and the other ultrastructural features of apoptosis. [all bars = 1μm]

a



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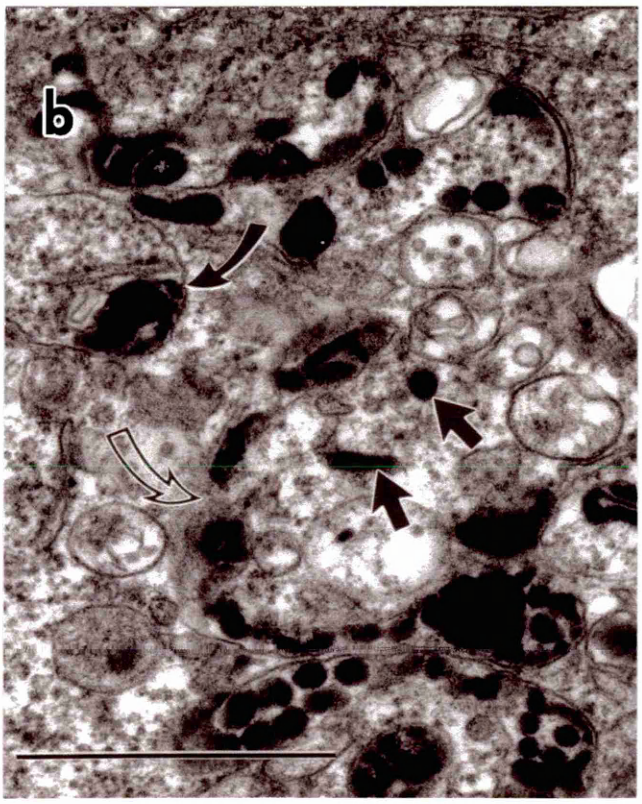

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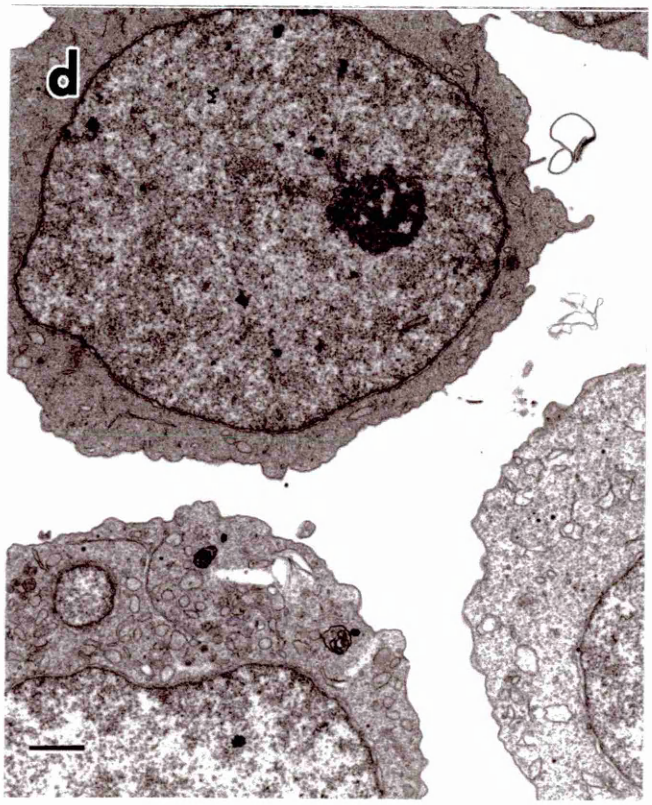


5.4 Bid cleavage is inhibited by Z-VAD.FMK

Cleavage of Bid is implicated in the release of mitochondrial cytochrome c in CD95-induced apoptosis (Li et al., 1998; Luo et al., 1998). This mechanism of cytochrome c release was investigated in order to determine whether it is important in chemical-induced apoptosis in THP.1 cells. Western blot analysis showed that Bid was present as an ~26 kDa protein in control cells and was cleaved in cells treated with etoposide (Fig. 18A). It was initially cleaved into two major fragments of ~ 15 and 14 kDa (Fig. 18A), most probably following cleavage at LQTD^{60}↓G and IEAD^{75}↓S (Li et al., 1998; Luo et al., 1998). Etoposide induced a time-dependent cleavage of Bid in THP.1 cells with a small amount of fragmentation first being observed at 3 h (Fig. 18A lane 4). Z-VAD.FMK inhibited this cleavage (Fig. 18A lane 6) but it did not inhibit etoposide-induced release of cytochrome c (Fig. 16A lane 7). Thus, in etoposide-induced apoptosis in THP.1 cells, release of mitochondrial cytochrome c did not involve the cleavage of Bid. TPCK also induced a time-dependent cleavage of Bid with the initial cleavage being observed at 2 h (Fig. 18B lane 3). Again, Z-VAD.FMK inhibited this cleavage (Fig. 18B lane 6) but failed to inhibit TPCK-induced release of cytochrome c (Fig. 16B lane 7). Thus, in both etoposide- and TPCK-induced cell death in THP.1 cells, cleavage of Bid was inhibited by Z-VAD.FMK, suggesting that this cleavage was not an essential event required for the release of mitochondrial cytochrome c in chemical-induced apoptosis.

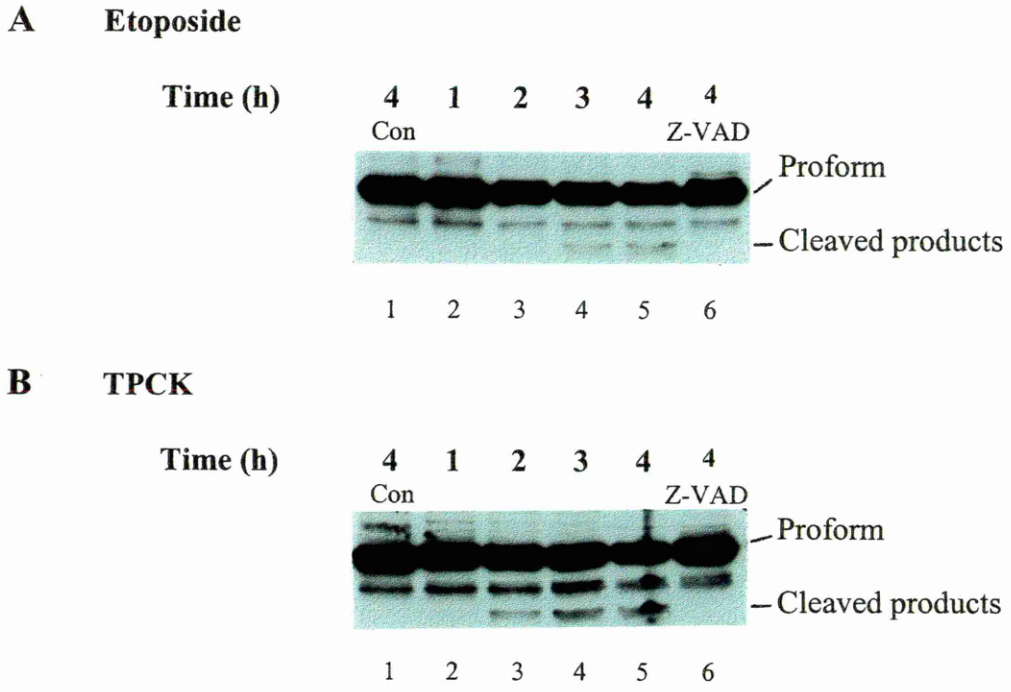


Figure 18. Z-VAD.FMK inhibits cleavage of Bid in both etoposide- and TPCK-induced apoptosis

THP.1 cells were incubated for the indicated times either alone (lane 1) or with (A) etoposide (25 μM) or (B) TPCK (75 μM) (lanes 2-5, respectively). Cells were also co-incubated with Z-VAD.FMK (50 μM) (lane 6). Cells were then analysed by Western blot analysis for Bid and its cleaved products as indicated.

5.5 Z-VAD.FMK inhibited TNF-α-mediated but not etoposide-induced cytochrome c release in U937 cells

The results described above demonstrated that Bid cleavage was not required for the release of mitochondrial cytochrome c in chemical-induced apoptosis in THP.1 cells although it may be important for cytochrome c release in cell surface death receptor-mediated apoptosis. In order to test this hypothesis, the study was extended to the human lymphoid tumour cell line U937. These cells are sensitive to TNF-α, which induces apoptosis through death receptor-mediated pathways (reviewed by Nagata, 1997). Both TNF-α/cycloheximide and etoposide induced apoptosis in U937 cells, as assessed by PS externalisation and decreased $\Delta\Psi_m$ (Fig. 19A). Induction of apoptosis was accompanied by processing of caspase-3 and cleavage of PARP, a commonly used measure of caspase-3-like enzymic activity (Fig. 19B and C, lanes 2 and 4). Z-VAD.FMK completely inhibited TNF-α/cycloheximide-induced apoptosis as assessed

by all these criteria. In contrast, in etoposide-induced apoptosis Z-VAD.FMK completely inhibited PS externalisation, the decrease in $\Delta\Psi_m$, and the cleavage of PARP but it only partially inhibited processing of caspase-3 (Fig. 19B lane 5). Thus Z-VAD.FMK was more effective at blocking the activity rather than the processing of caspase-3. Induction of apoptosis by either TNF-α/cycloheximide or etoposide also resulted in an increase in cytosolic cytochrome c (Fig. 19D lanes 2 and 4, respectively). Interestingly, Z-VAD.FMK blocked the release of cytochrome c from mitochondria in TNF-α/cycloheximide-mediated apoptosis but did not inhibit the increase in cytosolic cytochrome c in etoposide-induced apoptosis (Fig. 19D lanes 3 and 5, respectively). Thus, the data from U937 cells supported the hypothesis that Z-VAD.FMK inhibits a target upstream of mitochondria in receptor (TNF-α)-mediated apoptosis, whereas in etoposide-induced apoptosis the Z-VAD.FMK target is downstream of mitochondria. Cleaved Bid has been implicated in mediating the release of mitochondrial cytochrome c in receptor (CD95 and TNF-α)-induced apoptosis (Li et al., 1998; Luo et al., 1998) and this cleavage is inhibited by Z-VAD.FMK in CD95-induced apoptosis in Jurkat T cells (Luo et al., 1998). Thus, it is of particular interest to test whether Bid cleavage is also inhibited by Z-VAD.FMK in TNF-α-induced apoptosis in U937 cells. Induction of apoptosis by either TNF-α or etoposide resulted in the cleavage of Bid (Fig. 19E lanes 2 and 4, respectively). Z-VAD.FMK prevented cleavage of Bid induced by both apoptotic stimuli (Fig. 19E lanes 3 and 5, respectively). Because Z-VAD.FMK only inhibited cytochrome c release in TNF-α- but not in etoposide-induced apoptosis (Fig. 19D lanes 3 and 5, respectively), these results effectively ruled out the role of the Bid cleavage in cytochrome c release in etoposide-induced apoptosis, although it may play a critical role in cytochrome c release in TNF-α-induced apoptosis. Thus in both THP.1 and U937 cells, cleavage of Bid and loss of mitochondrial cytochrome c accompanied chemical-induced apoptosis and Z-VAD.FMK inhibited cleavage of Bid but not the loss of cytochrome c, demonstrating that cleavage of Bid is not responsible for the release of mitochondrial cytochrome c in chemical-induced apoptosis.

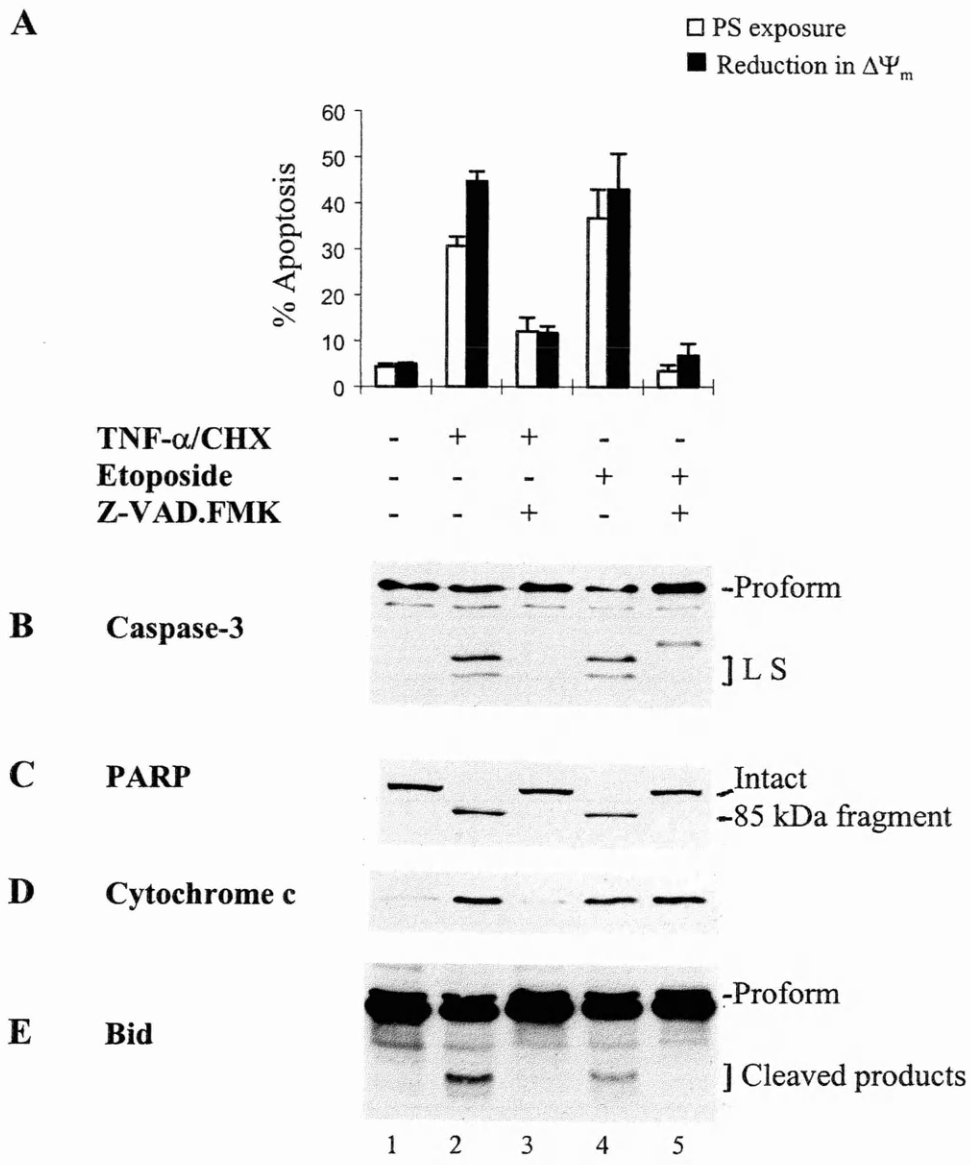


Figure 19. Z-VAD.FMK inhibits TNF-α- but not etoposide-induced release of mitochondrial cytochrome c in U937 cells

U937 cells were incubated for 3 h with TNF-α/cycloheximide (TNF/CHX) either alone (lane 2) or in the presence of Z-VAD.FMK (2 μM) (lane 3). Cells were also incubated for 5 h with etoposide either alone (lane 4) or in the presence of Z-VAD.FMK (20 μM) (lane 5). (A) Apoptosis was assessed flow cytometrically by PS exposureand decreased $\Delta\Psi_m$. Results were expressed as mean of at least three experiments. Cells were also analysed by Western blot analysis as described in Materials and Methods for, (B) processing of caspase-3, (C) cleavage of PARP (D) cytochrome c and (E) cleavage of Bid. Intact PARP (116 kDa) is cleaved by a caspase-3-like enzymic activity to an 89 kDa signature fragment. Intact Bid (26 kDa) is cleaved into two fragments of ~15 and 14 kDa.

5.6 Discussion

5.6.1 Release of cytochrome c is independent of the reduction in $\Delta\psi_m$

Release of mitochondrial cytochrome c together with caspase-9, Apaf-1 and ATP/dATP, forms a complex which then activates "effector" caspases such as caspase-3 (P. Li et al., 1997; Liu et al., 1996). Several recent studies have also highlighted a role for a decrease in $\Delta\psi_m$ as a key committed step in the induction of apoptosis (Zamzami et al.,1995; Petit et al., 1996). In the present study, both etoposide and TPCK caused a release of mitochondrial cytochrome c and a decrease in $\Delta\psi_m$ (Fig. 16A). Co-incubation with Z-VAD.FMK abolished the decrease in $\Delta\psi_m$ but did not affect the release of cytochrome c (Fig. 16 A and B lanes 7, respectively). These results demonstrate that the release of cytochrome c occurs upstream of the reduction in $\Delta\psi_m$. Further support for this conclusion was provided by the finding that TPCK-induced cytochrome c release was clearly observed at 1 h, prior to the reduction in $\Delta\psi_m$ or the increase in PS exposure (Fig. 16B). Thus, apoptosis induced by either etoposide or TPCK resulted in the release of mitochondrial cytochrome c, which occurred prior to or independent of the reduction in $\Delta\psi_m$, a result consistent with other studies (Kluck et al., 1997; Yang et al., 1997; Bossy-Wetzel et al.,1998).

5.6.2 Release of mitochondrial cytochrome c is upstream of the formation of ultracondensed mitochondria and discontinuities in the outer mitochondrial membrane

The transition of mitochondria from an orthodox to a condensed conformation is associated with a change from a resting (state 4) to an active (state 3) respiration (Hackenbrock, 1968; Lehninger, 1975). The characteristic increase in matrix density in the condensed conformation results from a reduction in the volume of the inner mitochondrial compartment, which is accompanied by a compensatory increase in the outer compartment/intracristal space resulting in little or no reduction in total volume of the organelle (Hackenbrock, 1968). The ultracondensed mitochondria described in the present study are distinct from the well-described condensed mitochondrial conformation and are characterised by a particularly pronounced increase in matrix density together with a marked decrease in overall volume (Fig. 17 a and b). The ultracondensed mitochondria in THP.1 cells exhibit similar features to those observed in apoptotic lymphoblastic leukemic cells (Jia et al., 1997), the "pleomorphic

micromitochondriosis" in apoptotic nodal myocytes (James et al., 1993) and the "mitochondrial pyknosis" and "hypercondensation" observed during apoptosis in a colon carcinoma cell line (Mancini et al., 1997). These changes were associated with an increased mitochondrial mass but with a decreased $\Delta\psi_m$ (Mancini et al., 1997). Despite minor differences in the morphology of these examples of highly condensed mitochondria, they all share a pronounced increase in matrix density together with a marked reduction in the area of their profiles in thin sections. Mitochondria resembling the classical condensed form have been described as ultracondensed in some instances of cell death (Papadimitriou et al., 1994). In some systems, there appears to be a progression from the condensed to the ultracondensed form followed by deletion of the organelle (Jia et al., 1997; Mancini et al., 1997). In the present study, co-incubation with Z-VAD.FMK resulted in mitochondria of normal appearance, with complete inhibition of the formation of ultracondensed mitochondria (Fig. 17c and d) without affecting the release of mitochondrial cytochrome c (Fig. 16 A and B). Thus formation of ultracondensed mitochondria occurred downstream of caspase activation.

In apoptotic THP.1 cells, many ultracondensed mitochondria showed rupture of the outer membrane (Fig. 17a and b) exposing regions of the condensed matrix to the cytosol. Mitochondria are susceptible to morphological change during delayed or inappropriate processing but control samples were devoid of any such condensation or discontinuities. Breaks in the outer mitochondrial membrane occur in the swollen mitochondria of apoptotic Jurkat and FL5.12 cells resulting in the redistribution of cytochrome c to the cytosol followed by a decrease of $\Delta\psi_m$ (Vander Heiden et al., 1997). These changes are prevented by expression of Bcl-x_L suggesting that it increases cell survival by modulating electrical and osmotic changes of mitochondria (Vander Heiden et al., 1997). In contrast, the present study shows ultracondensation rather than swelling of mitochondria in THP.1 cells before the development of any other morphological features of apoptosis. As Z-VAD.FMK inhibited the formation of outer mitochondrial membrane discontinuities (Fig. 17c and d), these changes were downstream of caspase activation. Thus our data have excluded two of the major hypotheses currently proposed for the release of mitochondrial cytochrome c, i.e. formation of outer mitochondrial membrane discontinuities and/ or resulting from a

decrease in $\Delta\psi_m$. An alternative mechanism for this release may be via a specific outer mitochondrial membrane channel. Bax, a proapoptotic member of the Bcl-2 family, forms channels in lipid membranes (Antonsson et al., 1997; Schlesinger et al., 1997) and has been proposed as a possible candidate for such a channel (Reed, 1997b). Our data support the hypothesis that an initial mitochondrial perturbation results in the release of cytochrome c, which in the presence of Apaf-1 leads to activation of caspases-9 and -3. The activated caspases then cleave cellular proteins resulting in mitochondrial damage leading to mitochondrial ultracondensation and outer membrane discontinuities.

5.6.3 Cytochrome c release occurs upstream of the activation of caspases in chemical-induced apoptosis

Following release from mitochondria, cytochrome c binds to Apaf-1 resulting in the activation of caspase-9, which in turn activates the effector caspases responsible for the cleavage of many of the substrates associated with the characteristic biochemical and morphological changes of apoptosis (P. Li et al., 1997). In agreement with data from chemical- and radiation-induced apoptosis in other cells (Kluck et al., 1997; Yang et al., 1997; Bossy-Wetzel et al., 1998), Z-VAD.FMK did not inhibit the release of mitochondrial cytochrome c in chemical-induced apoptosis in both THP.1 and U937 cells. However, Z-VAD.FMK blocked activation of caspases-3 and -7 and PARP cleavage (Fig. 19 B and C, and Fig. 12 in chapter 4). These data support the hypothesis that the target of Z-VAD.FMK in chemical-induced apoptosis is the processing or the activity of caspase-9, the initiator caspase after the release of cytochrome c. Recent studies on caspase-9 knockout mice demonstrate that caspase-9 is a critical upstream activator of a caspase cascade in vivo and in some situations is essential for the processing of caspase-3 (Kuida et al., 1998). Thus cytochrome c release occurs upstream of the activation of caspases.

Cleavage of Bid was observed in the present study in both chemical-induced and receptor-mediated apoptosis. Bid is cleaved by low concentrations of caspase-8 but can be cleaved by higher concentrations of caspase-3 (Li et al., 1998). Thus in chemical-induced apoptosis, inhibition of Bid cleavage by Z-VAD.FMK may be due to inhibition of processing/activity of caspase-3 after release of cytochrome c which,

in the presence of dATP and Apaf-1, resulted in the activation of caspase-9. Furthermore, in contrast to receptor-mediated apoptosis, Z-VAD.FMK blocked cleavage of Bid but not release of cytochrome c in chemical-induced apoptosis (Figs. 16, 18 and 19) demonstrating that cytochrome c release is a caspase-independent event and is not mediated by cleavage of Bid in these two models of chemical-induced apoptosis. Whether the mechanism of mitochondrial cytochrome c release involves another proapoptotic BH3 domain-containing member of the Bcl-2 family, such as Bik, Hrk, Blk or Bim remains to be seen. Thus in chemical-induced apoptosis, a mechanism other than cleavage of Bid is responsible for the release of cytochrome c.

Chapter 6

Role of Zn^{++} in apoptosis and necrosis

6.1 Introduction

The data obtained so far have demonstrated that activation of caspases is a key event during the execution phase of apoptosis. The cleavage of specific cellular proteins by some of the caspases appears to be associated with many of the morphological changes characteristic of apoptosis (reviewed by Kumar and Lavin, 1996; Nagata, 1997; Cohen, 1997). For example, proteolysis of nuclear proteins such as lamins (Kaufmann, 1989; Oberhammer et al., 1994), poly (ADP-ribose) polymerase (PARP) (Kaufmann, 1989; Kaufmann et al., 1993), DNA-dependent protein kinase (DNA-PK) (Casciola-Rosen et al., 1995; Song et al., 1996) and U1 small ribonucleoprotein (U1-70 kDa) (Casciola-Rosen et al., 1994; Tewari et al., 1995a) observed in cells undergoing apoptosis is believed to contribute to the collapse of the nucleus and condensation of the chromatin. Thus the biochemical machinery required to execute the apoptotic programme seems to be constitutively present in most mammalian cells, but regulation of the process can be modulated by a wide variety of extra- and intra-cellular factors (Steller, 1995; Wyllie, 1995). The potential role in the modulation of apoptosis by Zn^{++} has been known for many years (reviewed by Zalewski and Forbes, 1993; Sunderman, 1995; Fraker and Telford, 1997). Both *in vivo* and *in vitro* studies have shown that apoptosis is induced during Zn^{++} deficiency (Elmes, 1977; Dinsdale and Williams, 1977; Elmes and Jones, 1980; McCabe et al., 1993), whereas Zn^{++} supplementation inhibits apoptosis in a number of cell systems induced by diverse stimuli (Cohen and Duke, 1984; Shimuzu et al., 1991; Takano et al., 1991; Bicknell et al., 1994). Although the precise mechanisms of inhibition in apoptosis by Zn^{++} are largely unknown, several potential targets of Zn^{++} have been reported including a Ca^{++}/Mg^{++}-dependent endonuclease (Cohen and Duke, 1984; Giannakis et al., 1991) and the caspases (Wolf et al., 1997; Perry et al., 1997; Stennicke and Salvesen, 1997). However, the inhibitory effect of the cation on apoptosis was almost all observed following short time period of incubation. The question of what eventually happens to cells that are prevented from undergoing apoptosis has not been adequately addressed. In human monocytic THP.1 cells, the role of Zn^{++} in apoptosis has not been characterised. In the present study, the effects of Zn^{++} on apoptosis induced by either etoposide or TPCK were examined with particular interest in its effect after longer incubation.

6.2 Concentration-dependent inhibition of apoptosis by Zn^{++}

Incubation of THP.1 cells for 4 h with either etoposide (25 μM) or TPCK (75 μM) resulted in the induction of apoptosis, as assessed by the externalisation of PS (Fig.20 A). Zn^{++} (0.1 mM) had no inhibitory effect on the PS externalisation in cells treated with etoposide but partly inhibited that in cells treated with TPCK. Zn^{++} (0.5 and 1.0 mM) completely inhibited the PS externalisation in cells treated with either etoposide or TPCK. Thus Zn^{++} inhibited, in a concentration-dependent manner, the induction of apoptosis induced by either agent (Fig. 20A). Zn^{++} alone at all three concentrations did not induce PS externalisation (Fig. 20A, lanes 12-14). Z-VAD.FMK (50 μM), the inhibitor of caspases, also inhibited apoptosis induced by the two apoptotic stimuli (Fig. 20A). The induction of apoptosis by either etoposide or TPCK was accompanied by the activation of caspases together with ensuing proteolysis of PARP and lamins (Fig. 10 in chapter 3, and MacFarlane et al., 1997; Zhu et al., 1997). This proteolysis together with the cleavage of DNA by etoposide or TPCK was prevented by the treatment with Z-VAD.FMK (Figs. 12 and 13 in chapter 4; and MacFarlane et al., 1997; Zhu et al., 1997). The effect of Zn^{++} on these changes associated with the induction of apoptosis was therefore investigated in THP.1 cells.

First, the effect of Zn^{++} on the internucleosomal cleavage of DNA induced by etoposide was examined using conventional agarose gel electrophoresis, and Zn^{++} exhibited a concentration-dependent inhibition on the formation of internucleosomal fragments of DNA (data not shown). The effects of Zn^{++} on the activities of caspases, including cleavage of PARP and lamin B1 was then examined using Western blot analysis. Figure. 20 B and C showed that control cells (lane 1) contained only intact PARP (116 kDa) and lamin B1 (~67 kDa) respectively. Induction of apoptosis by either etoposide or TPCK was accompanied by the cleavage of PARP to its 85 kDa fragment (Fig. 20 B lanes 2 and 7) and of lamin B1 to its ~46 kDa fragment (Fig. 20 C lanes 2 and 7), respectively, which were all completely blocked by Z-VAD.FMK (Fig. 20 B and Fig. 20 C lanes 6 and 11, respectively). Zn^{++} inhibited, in a concentration-dependent fashion, the cleavage of PARP and lamin B1 (Figs. 20 B and C, lane 3-5 and 8-10) in cells treated with either apoptotic stimulus whilst Zn^{++} alone at all three concentrations did not induce the cleavage of either PARP (Fig. 20 B lanes 12-14) or lamin B1 (Fig. 20 C lanes 12-14). It was noted that 1 mM Zn^{++} , while clearly

preventing the cleavage of PARP after etoposide treatment (Fig. 20 B lane 5), did not completely block that after TPCK treatment (Fig.20 B lane 10).

The cleavage of PARP to its 85 kDa signature fragment suggested the activation of caspases-3 and/or -7 whereas that of lamin B1 indicated the activation of caspase-6. As it has been shown that Zn^{++} inhibited the cleavage activity of caspase-6 (Takahashi et al., 1996), it was decided to determine if Zn^{++} had any inhibitory effect on the activation of caspases-3 and -7. Untreated control cells showed the presence of the intact proforms of caspase-3 (Fig. 20 D lane 1) and caspase-7 (Fig. 20 E lane 1). Induction of apoptosis with either etoposide or TPCK was accompanied by a decrease in the proforms of caspases-3 and -7 together with the formation of their respective catalytically active large subunits (LS) (Figs. 20 D and E, lanes 2 and 7, respectively). Zn^{++} (0.1 mM) showed no inhibitory effect on the formation of LS of caspase-3 in cells treated with either etoposide (Fig. 20 D lane 3) or TPCK (Fig. 20 D lane 8). Zn^{++} (0.5 mM) almost completely inhibited the formation of LS of caspase-3 in etoposide-treated cells (Fig. 20 D lane 4), but only partly inhibited the formation of LS of caspase-3 in TPCK-treated cells (Fig. 20 D lane 9). Zn^{++} (1.0 mM) completely inhibited the formation of LS of caspase-3 in cells treated with etoposide (Fig. 20 D lane 5) and largely inhibited that in cells treated with TPCK (Fig. 20 D lane 10). Zn^{++} showed a similar inhibitory pattern in the formation of LS of caspase-7 following treatment with either apoptotic stimulus (Fig. 20 E). Thus Zn^{++} inhibited, in a concentration-dependent manner, the formation of LSs of caspases-3 and -7 in cells treated with either etoposide or TPCK. Zn^{++} alone, at all three concentrations, did not induce the formation of LS of either caspase-3 (Fig. 20 D lanes 12-14) or caspase-7 (Fig. 20 E, lanes 12-14). Z-VAD.FMK clearly blocked the formation of LSs of caspase-3 and -7 after treatment with either etoposide or TPCK (Figs. 20 D and E, lanes 6 and 11, respectively). Taken together, these results suggested that Zn^{++} inhibited apoptosis by blocking the processing of both caspase-3 and -7 to form their respective catalytically active large subunits. While this work was in progress, it was reported that Zn^{++} was a potent inhibitor of caspase-3 as it inhibited both processing/activation and activity of the protease (Wolf et al., 1997 and Perry et al., 1997). Further study showed that Zn^{++} was a general inhibitor of caspases, although

their sensitivities to Zn^{++} vary significantly (Stennicke and Salvesen, 1997). Thus the data from this study are in good agreement with these observations.

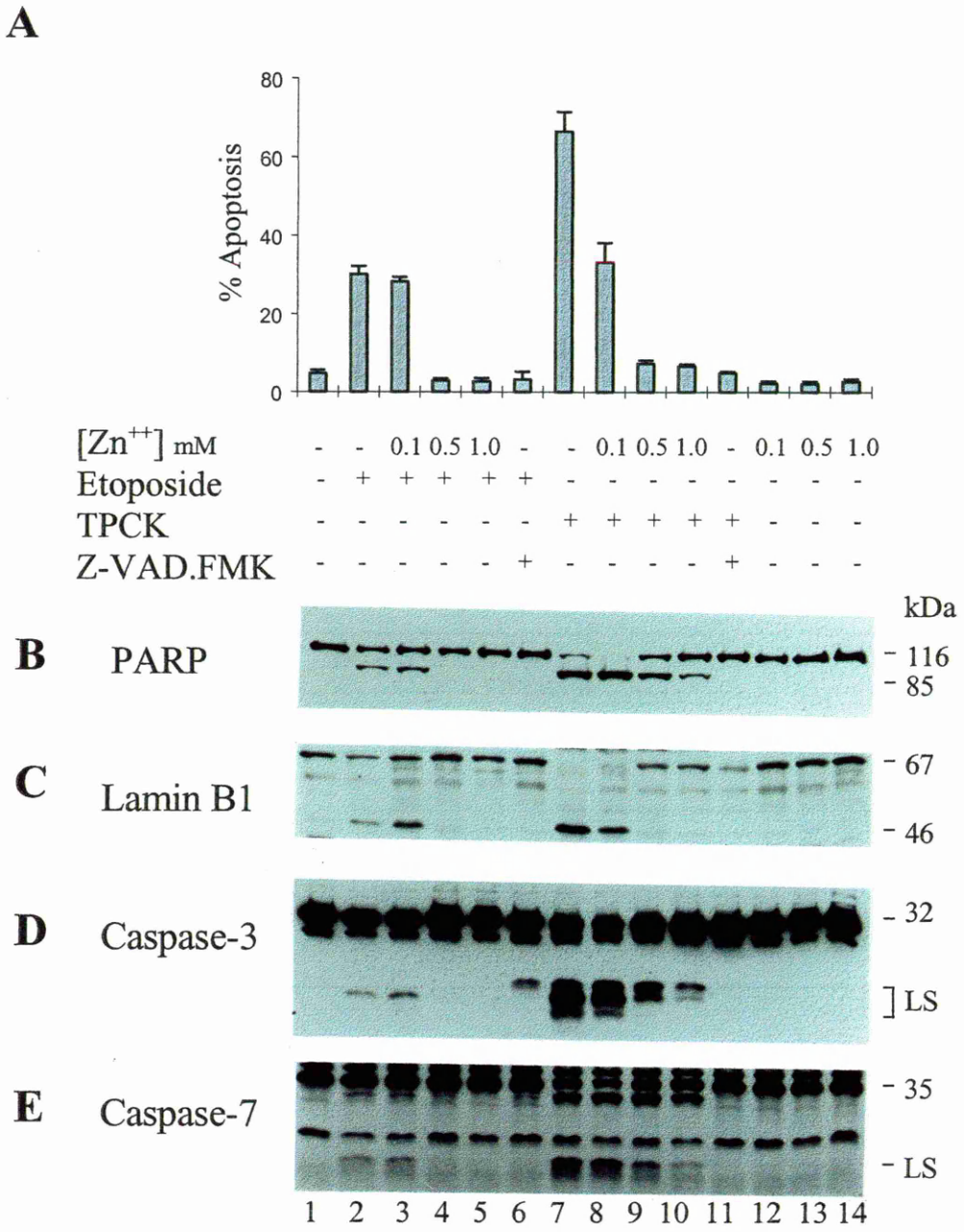


Figure 20. Z-VAD.FMK and Zn^{++} inhibit PS exposure, cleavage of PARP and lamin B1 and processing of caspases-3 and -7

THP.1 cells were incubated for 4 h either alone (lane 1), or with etoposide (25 μM) (lanes 2 - 6) or TPCK (75 μM) (lanes 7 - 11) in the presence of Z-VAD.FMK (50 μM) (lanes 6 and 11) or Zn^{++} (0.1-1 mM) (lanes 3 - 5 and 8 -10) as indicated. Cells were also incubated with Zn^{++} alone (0.1-1 mM) (lanes 12-14). (A) The induction of apoptosis was assessed by externalisation of PS as described in Materials and Methods. Cells were examined by Western blot analysis using antibodies to detect the cleavage of (B) PARP and (C) lamin B1. Cells were also analysed for the cleavage of the (D) 32-kDa procaspase-3 and (E) 35-kDa procaspase-7 to their respective catalytically active large subunits (LS). Results were expressed as mean ± sem of at least three experiments or representative of three experiments.

6.3 Zn^{++} inhibited the release of cytochrome c in etoposide- but not TPCK-induced apoptosis

It has previously been shown that cytochrome c is released from mitochondria during the induction of apoptosis by either apoptotic stimulus (see Fig. 16 in chapter 5). A reduction in mitochondrial membrane potential ($\Delta\Psi_m$) has also been observed as an event associated with apoptosis (see Fig. 8 in chapter 3). Experiments were therefore performed to investigate whether Zn^{++} could inhibit the release of cytochrome c and reduction in $\Delta\Psi_m$ as well. Incubation with etoposide resulted in a time-dependent accumulation of cytochrome c in cytosolic extracts, detected by western blot analysis (Fig. 21 lanes 2-6, *upper blot*). Whilst Z-VAD.FMK did not block cytochrome c release induced by etoposide, Zn^{++} (1 mM) blocked its release into cytosol (Fig. 21 compare lanes 8 with 7, *upper blot*). Zn^{++} (1 mM) alone did not cause the release of cytochrome c (see below). Zn^{++} also caused a concentration-dependent inhibition of the reduction in $\Delta\Psi_m$ when co-incubated with etoposide whereas it alone at all three concentrations had no effect on $\Delta\Psi_m$ (Table 4). Thus these data have shown that Zn^{++} inhibited several features of apoptosis including the release of cytochrome c and reduction in $\Delta\Psi_m$ following treatment with etoposide. However, further experiments showed that neither Zn^{++} (1 mM) nor Z-VAD.FMK blocked the release of cytochrome c during the induction of apoptosis by TPCK (Fig. 21 lanes 7 and 8, *lower blot*), although they both inhibited the reduction in $\Delta\Psi_m$ (Table 4). These data thus showed that Zn^{++} inhibited etoposide- but not TPCK-induced release of cytochrome c during induction of apoptosis.

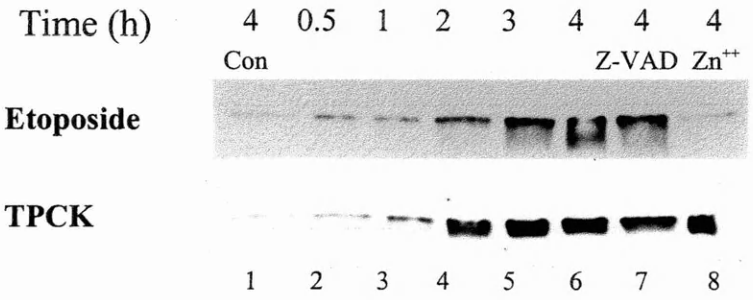


Figure 21. Zn^{++} inhibited the release of cytochrome c in etoposide-induced apoptosis

Cells were incubated for the indicated times either alone (lane 1) or with etoposide (25 μM) or TPCK (75 μM) (lanes 2 - 6). Cells were also co-incubated with Z-VAD.FMK (50 μM) (lane 7) or Zn^{++} (1 mM) (lane 8). Cytosolic extracts were prepared and assessed by Western blot analysis for cytochrome c as described in Material and Methods. No detectable cytochrome oxidase subunit II, a marker of mitochondrial contamination, was observed in these samples (data not shown).

Table 4. Zn^{++} and Z-VAD.FMK inhibit the decrease of $\Delta\Psi_m$ during the induction of apoptosis

Treatment	Zn^{++} (mM)	% cells with low $\Delta\Psi_m$
Control	-	6.1 ± 0.8
Etoposide	-	24.4 ± 1.2
Etoposide	0.1	23.4 ± 2.5
Etoposide	0.5	11.4 ± 4.7
Etoposide	1.0	7.1 ± 1.8
Etoposide + Z-VAD.FMK	-	3.9 ± 0.7
TPCK	-	44.2 ± 4.6
TPCK	1.0	9.5 ± 1.7
TPCK + Z-VAD.FMK	-	5.6 ± 1.1
Zn^{++}	0.1	6.4 ± 1.7
Zn^{++}	0.5	5.9 ± 1.2
Zn^{++}	1.0	8.2 ± 2.7
Z-VAD.FMK	-	2.6*
m-ClCCP	-	79.8 ± 2.9

THP.1 cells were incubated with etoposide (25 μM) or TPCK (75 μM) for 4 h either alone or in the presence of the indicated concentrations of Zn^{++} or Z-VAD.FMK (50 μM). The percentage of cells with decreased mitochondrial membrane potential ($\Delta\Psi_m$) was determined by flow cytometry. Cells were also incubated with the mitochondrial uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (m-ClCCP, 50 μM) as a positive control. The results are expressed as the mean ± sem of at least three separate experiments except for * , which was the average of two experiments.

6.4 Zn^{++} did not prevent subsequent cell death despite inhibition of apoptotic pathways

Early work in this laboratory has shown that, in the presence of Zn^{++}, apoptosis proceeded without the internucleosomal cleavage of DNA in thymocytes following 4 h incubation with either etoposide or dexamethasone (G. Cohen et al., 1992; Brown et al., 1993; Sun et al., 1994), but was inhibited in human leukaemic U937 cells during a 7 h incubation with actinomycin D (Bicknell et al., 1994). To answer the question whether Zn^{++} prevents or merely delays apoptosis in THP.1 cells, the incubation time was extended to 24 h and cells were then examined for the activation of caspase-7 and PARP cleavage in cells treated with etoposide. Figure 22 A showed that at 24 h control cells retained the intact caspase-7 proform (lane 1) whilst cells treated with etoposide (25 μM) had a lower amount of proform with the formation of a corresponding catalytically active large fragment (lane 2). Zn^{++} still inhibited the activation of caspase-7 in a concentration-dependent manner whereas it alone at all three concentrations did not induce the formation of large fragment (Fig. 22 A lanes 7-9). Z-VAD.FMK (50 μM) effectively blocked the activation of caspase-7 (Fig. 22 A lane 6) whilst it alone had no effect (Fig. 22 A lane 10). Control cells contained only the intact form of PARP (Fig. 22 B lane 1) whereas etoposide-treated cells almost completely lost the intact form with the subsequent formation of the 85 kDa fragment (Fig. 22 B lane 2). Zn^{++} also inhibited the PARP cleavage induced by etoposide in a concentration-dependent manner whereas Zn^{++} alone at all three concentrations had no effect (Fig. 22 B). Z-VAD.FMK clearly inhibited the cleavage of PARP (Fig. 22B lane 6) whereas it alone showed no effect (Fig. 22 B lane 10). Thus the data indicated that Zn^{++} still effectively blocked the activation of caspase-7 and the proteolytic activities of caspases responsible for PARP cleavage after 24 h co-incubation with etoposide. However, when the cell viability was examined at 24 h, by trypan blue staining, most of the cells treated with etoposide and those co-incubated with Zn^{++} had lost their viability (Fig. 22 C). Surprisingly, cells treated with Zn^{++} alone also showed a concentration-dependent loss of viability. Partial loss of viability was also observed in cells co-incubated with etoposide and Z-VAD.FMK. Only control cells and those treated with either 0.1 mM Zn^{++} or 50 μM Z-VAD.FMK alone maintained greater than 95 % viability (Fig. 22 C). These results suggested that cells were still undergoing cell death when co-incubated with etoposide and Zn^{++},

even though caspase-7 activation and PARP cleavage were inhibited. They also indicated that Zn^{++} itself might exhibit a cytotoxic effect at the concentrations which inhibited apoptosis. More importantly, these data showed that Zn^{++} did not protect cells from subsequent cell death.

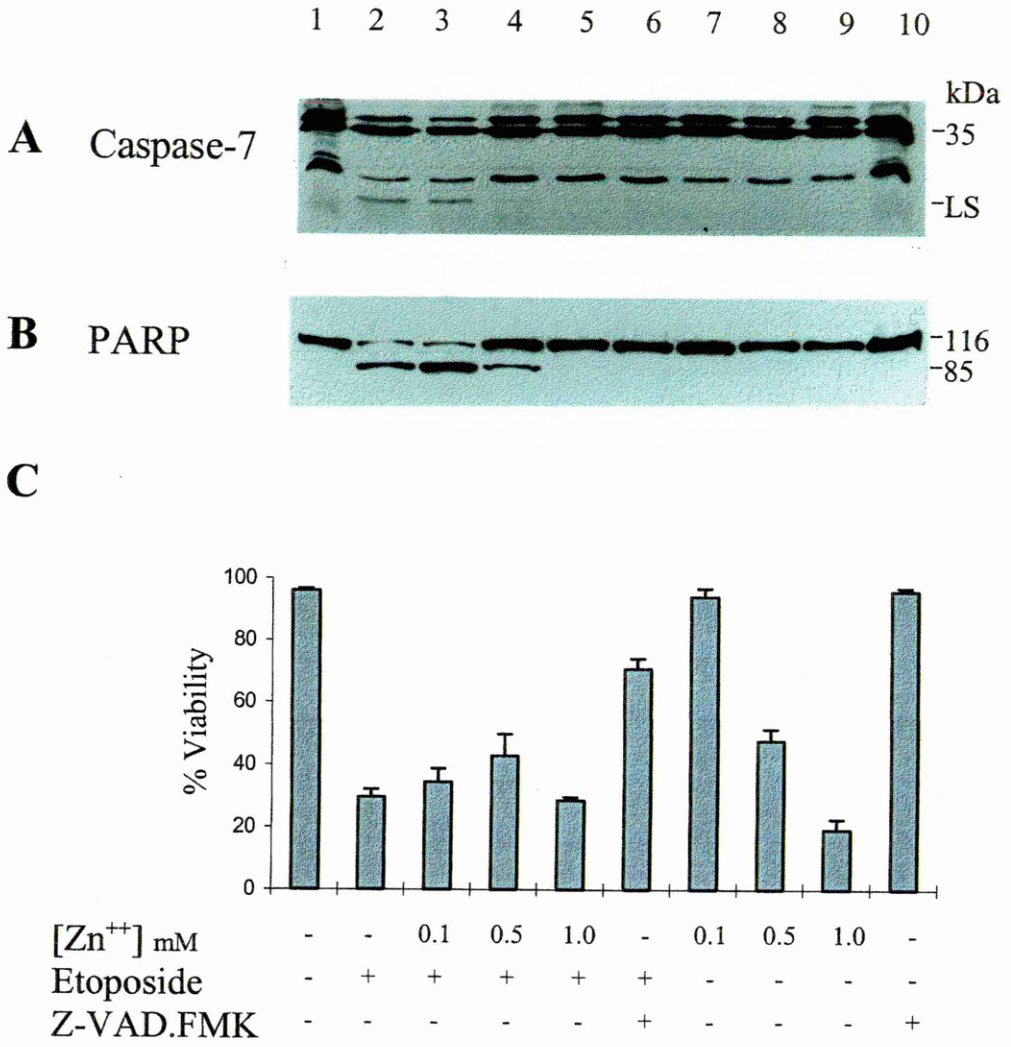


Figure 22. Zn^{++} did not prevent subsequent cell death despite inhibition of apoptotic pathways

THP.1 cells were incubated for 24 h either alone (lane 1), or with etoposide (25 μM) (lanes 2 - 6) in the presence of Z-VAD.FMK (50 μM) (lanes 6) or Zn^{++} (0.1-1 mM) (lanes 3-5). Cells were also incubated with Zn^{++} alone (0.1-1 mM) (lanes 7-9) or Z-VAD.FMK alone (lane 10). Cells were then analysed by Western blot analysis using antibodies to (A) 35-kDa pro-caspase-7 and its catalytically active large subunits (LS), and (B) intact PARP and cleaved fragment. (C) Cell viability at 24 h was examined by trypan blue staining. Results were expressed as mean ± sem of at least three experiments or representative of three experiments.

6.5 Longer incubation with Zn^{++} alone resulted in necrosis

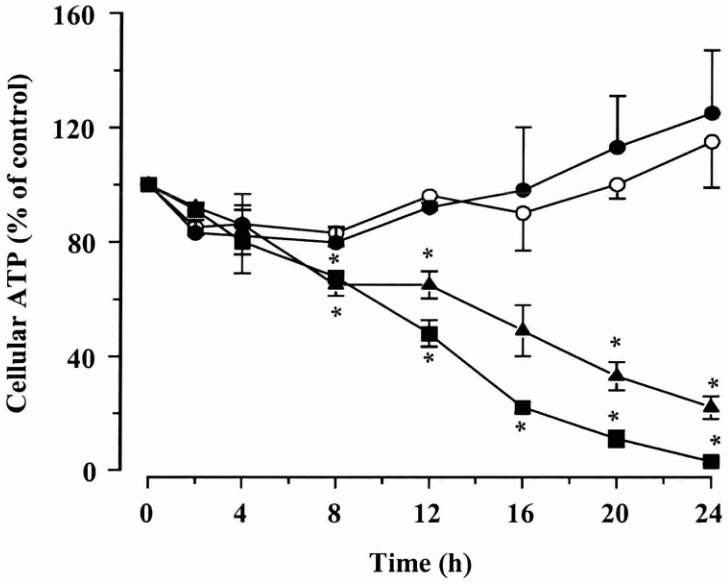
In order to characterise the cell death caused by Zn^{++}, time-course experiments were carried out in cells treated with Zn^{++} alone within the range of concentrations that inhibit apoptosis. Since the cells dying after 24 h incubation with Zn^{++} showed no sign of either caspase-7 activation or PARP cleavage (Fig. 22 A and 3B), it was assumed that the cell death caused by Zn^{++} was most probably by necrosis. It was therefore decided to monitor the intracellular ATP levels and the cell viability, the latter being assessed by the cell's uptake of propidium iodide. Zn^{++} (0.1 mM) had no effect on the intracellular ATP levels compared with control (Fig. 23 A) . Zn^{++} (0.5 mM) induced a decrease in the intracellular ATP levels at 8 h and showed a continuing time-dependent decrease (Fig. 23 A). The decrease in ATP levels caused by Zn^{++} (1.0 mM) also occurred after 4 h incubation and became more pronounced with time and by 24 h ATP levels dropped to lower than 5 % compared with that at the start of treatment (Fig. 23 A). Correlating well with the decrease in ATP levels was the loss of cell viability. Zn^{++} (0.5 mM) caused a loss of cell viability at 12 h and in a time-dependent manner thereafter (Fig. 23 B) and with Zn^{++} (1.0 mM) the loss of cell viability was apparent after 8 h and by 24 h more than 70 % of the cells lost their viability. In contrast, cells treated with 0.1 mM Zn^{++} showed almost identical viability to control cells (Fig. 23 B). Thus the results demonstrated that Zn^{++} alone at concentrations that inhibit apoptosis can cause cell death over a 24 h incubation period, which can be characterised by the decrease in intracellular ATP levels and the loss of cell viability. Ultrastructural studies revealed no signs of injury at 4 h (data not shown) whereas at 8 h cells were swollen. This was accompanied by a reduction in the density of the cytoplasm together with slight swelling of the lysosomes, endoplasmic reticulum and nuclei (Fig. 23 C). A few of these cells showed the development of flocculent densities within their mitochondria (Fig. 23 C). At 12 h these changes were more widespread and accompanied by chromatolysis or the clumping of heterochromatin into complex, densely-staining structures which were generally isolated from the inner membrane of the nuclear envelope (Fig. 23 D). By 24 h most cells were clearly in the late stages of necrosis exhibiting flocculent densities in the mitochondrial matrix, chromatolysis and lysis of many organelles (data not shown). Thus the necrotic cells observed 24 h after treatment with Zn^{++} have died by oncosis, a process distinct from apoptosis (Majno and Joris 1995).

Oncosis is characterised by a loss of cellular ATP and cellular swelling leading to the formation of necrotic cells. In order to determine at what stage these cells were irreversibly committed to die, cells were incubated with Zn^{++} (1 mM) for short periods of time and then transferred to a Zn^{++} - free medium. At 24 h no loss of viability was observed when Zn^{++} was removed after 2 h incubation, whereas 50 % of cells died when Zn^{++} was removed after 4 h incubation (see below). Thus cells were irreversibly committed to die as early as 4 h after exposure to Zn^{++}, prior to the development of detectable ultrastructural changes.

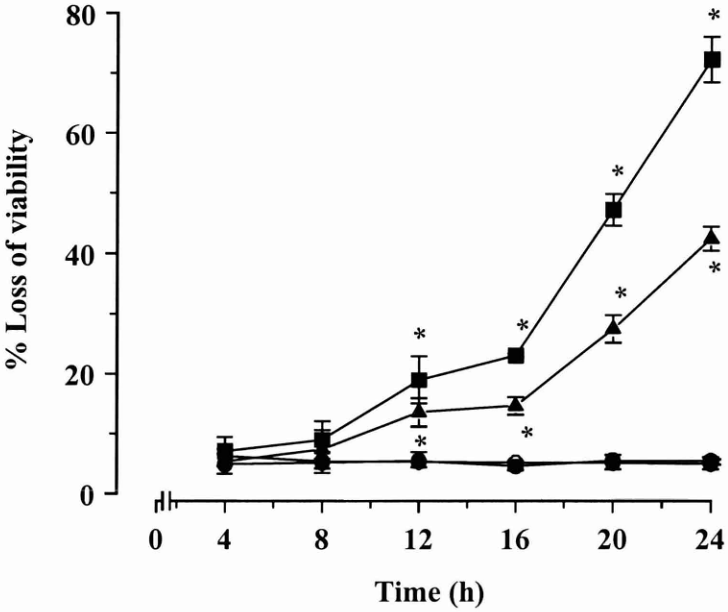
Figure 23. Zn^{++} caused time- and concentration-dependent depletion of ATP, loss of viability and ultrastructural changes of necrosis.

THP.1 cells were incubated for up to 24 h either alone (-o-), or with Zn^{++} at the concentrations of 0.1 mM (-●-) or 0.5 mM (-▲-) or 1.0 mM (-■-). (A) relative ATP levels were measured at the indicated time points as described in Materials and methods. The level of ATP in the control cells at zero time was 9.0 ± 0.6 nmoles / 10^6 cells. (B) % loss of viability was measured by uptake of propidium iodide by flow cytometry. Results are expressed as the mean ± sem of at least three experiments. The data were analysed by Student's *t* test and differences between control and treatment of $p<0.05$ were considered significant (*). (C) Incubation with Zn^{++} (1 mM) for 8 h, resulted in a slight reduction in cytoplasmic density, swelling of lysosomes, endoplasmic reticulum and nuclei together with the clumping of heterochromatin and the development of flocculent densities within mitochondria (fine arrows). (D) Incubation with Zn^{++} (1 mM) for 12 h, resulted in a marked increase in the density of the clumped chromatin, reduction in cytoplasmic density, discontinuities in the cell membrane (solid arrow) and lysis of many of the organelles, including mitochondria. [all bars = 1μm)

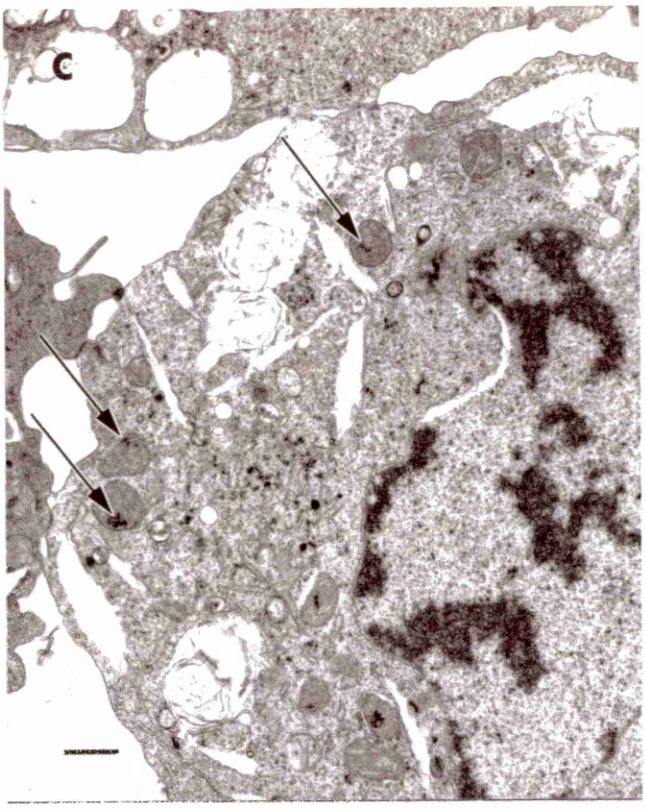
A


Cellular ATP (% of control)
160
120
80
40
0
0
4
8
12
16
20
24
Time (h)

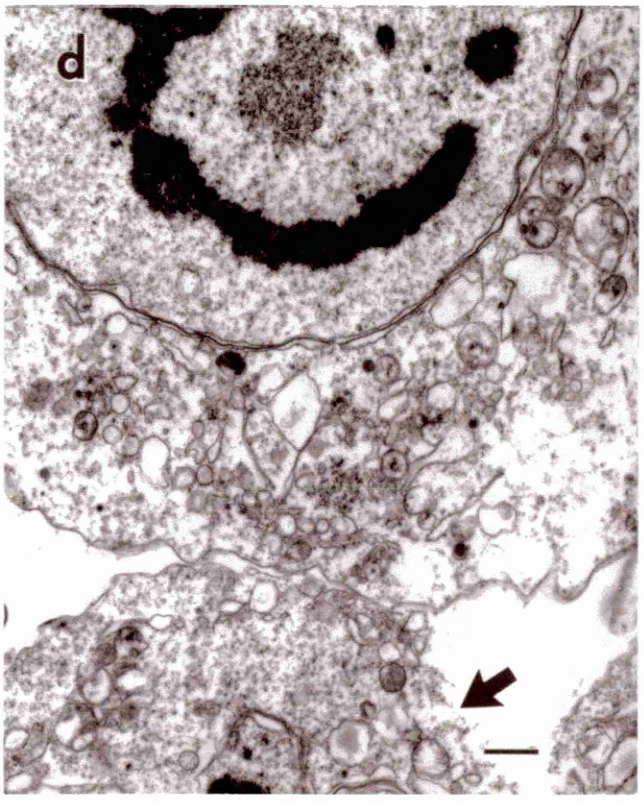
B


% Loss of viability
80
60
40
20
0
0
4
8
12
16
20
24
Time (h)

C



D



6.6 Cytochrome c release was a late event in non-apoptotic cell death

While many recent studies have concentrated on a possible role of cytochrome c release in apoptosis, few if any have investigated a possible relationship between this release and other forms of cell death. As Zn^{++} (1mM) caused cell death by oncosis over a 24 h incubation period, it was of interest to determine when cytochrome c was released in this non-apoptotic form of cell death. Zn^{++} (1mM) alone induced a time dependent increase in cytosolic accumulation of cytochrome c, which was first observed at 12 h (Fig. 24), after the loss of intracellular ATP (Fig. 23 A) and ultrastructural changes which were observed at 8 h (Fig. 23 C). Thus release of mitochondrial cytochrome c was a late event in oncosis and does not play a significant role in this non-apoptotic form of cell death.

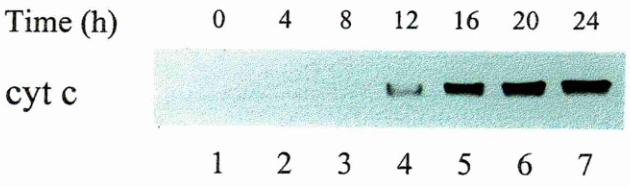


Figure 24. Cytochrome c release is a late event in necrosis

Cells were incubated with Zn^{++} (1mM) for up to 24 h. Cytosolic extracts were prepared at the indicated time points and assessed for cytochrome c (cyt c) by western blot analysis as described in Materials and methods.

6.7 Discussion

6.7.1 Zn^{++} is a potent inhibitor of caspases

The data presented in this study demonstrate the inhibitory effects of Zn^{++} on apoptosis characterised by the activation of caspases and their proteolytic activity, PS exposure, reduction in $\Delta\psi_m$, and DNA fragmentation. The broad spectrum of inhibition suggest the possible existence of multiple sites of action by Zn^{++} on the apoptotic pathways, including some early events during the execution phase. The data and that of others have clearly shown that Zn^{++} is a potent inhibitor of caspases (Wolf et al., 1997; Perry et al., 1997; Stennicke and Salvesen, 1997). It has been reported that cytochrome c is required for the activation of caspases (Liu et al., 1996) and the finding from the present study that Zn^{++} blocked the release of cytochrome c into the

cytosol in etoposide-induced apoptosis also suggested that Zn^{++} may interfere with the initial activation of caspases so preventing the progression of apoptotic protease cascade. Thus these results are consistent with the observation that cytochrome c is an essential component of the complex which initiates this cascade (Li et al., 1997). The possibility that Zn^{++} interferes with the transduction of the apoptotic signal prior to the release of cytochrome c may not be ruled out, but it is unlikely as Zn^{++} does not block the release of cytochrome c during TPCK-induced apoptosis although it inhibits all the other apoptotic features. The mechanism by which Zn^{++} blocks the release of cytochrome c in etoposide- but not in TPCK-induced apoptosis is still unresolved. As Zn^{++} inhibits both the activation of caspases and decrease in $\Delta\psi_m$ but does not block the release of cytochrome c in TPCK-induced apoptosis (Table 4 and Fig. 21, *lower blot*), it provides further evidence that release of cytochrome c occurs upstream of the activation of caspases and also prior to the decrease in $\Delta\psi_m$.

6.7.2 Mechanism of non-apoptotic cell death induced by Zn^{++}

Zn^{++} alone induces a non-apoptotic cell death, consistent with the process of cell death normally referred to as oncosis (Majno and Joris, 1995; Trump et al., 1997). Irreversible commitment of these cells to die occurs at 4 h, prior to the release of cytochrome c. Thus this release is a late event in the oncotic death of THP.1 cells. The characteristic depletion of ATP in oncotic cells may preclude the activation of caspases, whereas in apoptotic cells, early release of mitochondrial cytochrome c following interaction with Apaf-1 and dATP results in the activation of caspases, so precipitating the biochemical and morphological features of apoptosis. The mode of cytotoxicity of Zn^{++} appears to be non-specific because it does not cause immediate disruption of cellular function as the intracellular ATP levels during the first 4h incubation were similar to that of control cells (Fig. 23 A). Also, when cells were incubated with Zn^{++} (1 mM) for up to 2h, then washed to remove excess Zn^{++} and re-suspended in fresh Zn^{++}-free medium they maintained normal growth showing neither reduction in intracellular ATP levels nor decrease in cell viability at 24h (Fig. 25). Only those cells being exposed to Zn^{++} for 4 h exhibited significant losses in both intracellular ATP levels and cell viability, suggesting that cells became irreversibly committed to die at between 2 h and 4 h following exposure to Zn^{++}. The potential targets of Zn^{++} in the cytotoxic action includes calcium antagonism, zinc finger

proteins, microtubules and disruption in mitotic phase of cell cycle (reviewed by Zalewski and Forbes, 1993). Thus, depending on the cell type, Zn^{++} may exert divergent effects from inhibiting apoptotic pathways to inducing cell death. Therefore, in THP.1 cells Zn^{++} does not prevent cell death, a result consistent with the previous observation in this laboratory (G. Cohen et al., 1992) and those of others (Barbieri et al., 1992; McCabe et al., 1993). In addition, Zn^{++} alone at the concentration that inhibits apoptosis causes necrosis, in agreement with a finding of the other study (McGowan et al., 1994).

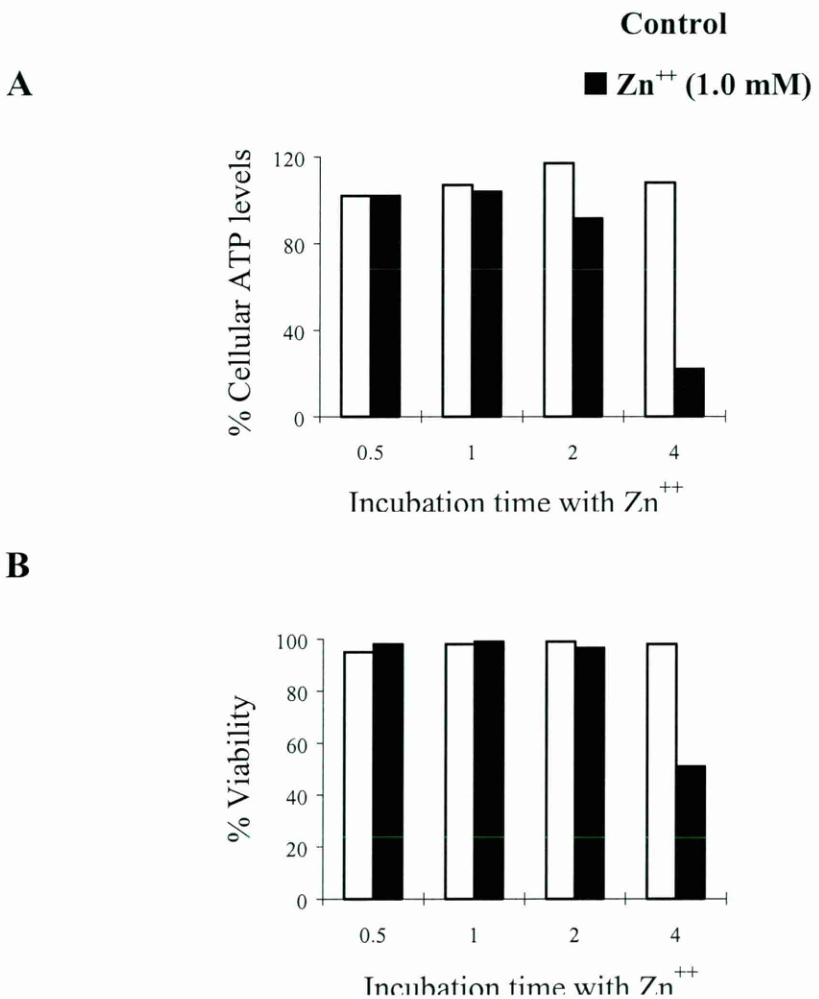


Figure 25. Short exposure (up to 2 h) to Zn^{++} caused neither decrease in intracellular ATP levels nor loss of cell viability at 24 h

THP.1 cells were incubated either alone or with Zn^{++} (1.0 mM) for the indicated time periods and then washed to remove excess Zn^{++} and re-suspended in Zn^{++}-free medium for continued incubation for up to 24 h. At 24 h, (A) relative intracellular ATP levels (% of control cells at zero time point) were determined as described in Materials and methods. (B) Cell viability at 24 h was also measured by trypan blue staining.

The observation that Zn^{++} alone at the concentration that inhibited apoptosis caused necrosis over an extended incubation period certainly poses a dilemma to the attempt of using Zn^{++} for therapeutic gain. It also provides a cautious note on studies of apoptosis using inhibitors. These studies should not only look at the effect of inhibition of apoptosis in a short time period but also examine the effect of the inhibitor on cell survival over an extended time period and also investigate whether inhibitor itself induces cell death. This is to ensure that inhibitor of apoptosis provides cells with long-term protection but not just a temporary blockade of certain changes associated with apoptotic phenotype. Theoretically, inhibitors of apoptosis should allow cells to survive and proliferate in particular if they are to be used therapeutically to inhibit the apoptotic process.

Chapter 7

General discussion

7.1 Caspases act upstream of the reduction in $\Delta\psi_m$

In this study, apoptosis was induced in THP.1 cells by two chemicals, the anti-cancer drug, etoposide, and the serine protease inhibitor, TPCK. Apoptosis, characterised morphologically by the condensation of the cytoplasm and the nucleus as well as chromatin fragmentation, was accompanied by activation of caspases-3 and -7 together with the cleavage of PARP and lamins, reduction in $\Delta\psi_m$, PS externalisation and DNA fragmentation. Several recent studies have highlighted a role for a decrease in $\Delta\psi_m$ as a key committed step in the induction of apoptosis (Zamzami et al., 1995; Castedo et al., 1996). However, several lines of evidence obtained from the present study support the notion that caspases act upstream of the reduction in $\Delta\psi_m$. Firstly, the time-course experiments demonstrated that during the induction of apoptosis activation of caspase-7 was evident at 1 h whereas both reduction in $\Delta\psi_m$ and increased PS exposure were first observed at 2 h after treatment with TPCK (compare Fig. 10 B with Fig. 8 B in chapter 3). Secondly, Z-VAD.FMK, the broad spectrum caspase inhibitor, blocked the activation of caspases, PS exposure and reduction in $\Delta\psi_m$ during the induction of apoptosis by either etoposide or TPCK in THP.1 cells (Figs. 11 and 12 in chapter 4). It also inhibited the activation of caspases, PS exposure and reduction in $\Delta\psi_m$ during the induction of apoptosis by TNF-α or etoposide in U937 cells (Figs. 15 in chapter 4 and 19 in chapter 5). Therefore, in these models of apoptosis, activation of caspases occurs upstream of a decrease in $\Delta\Psi_m$. This observation is consistent with some recent studies of apoptosis induced by staurosporine, UV irradiation or etoposide in several different cell systems (Yang et al., 1997; Kluck et al., 1997; Bossy-Wetzel et al., 1998), supporting the role of caspases as the key execution machinery in apoptosis. In addition, the data from this study and others suggest that the reduction in $\Delta\psi_m$ may be a consequence of caspase activity and regulated by the caspases. Caspases have been shown to be able to directly induce the disruption in $\Delta\psi_m$ in either isolated mitochondria (Susin et al., 1997; Marzo et al., 1998a) or the PT pore complex reconstituted in liposomes (Marzo et al., 1998b). In some cases where apoptosis is induced through the engagement of the death receptors (e.g. CD95), expression of Bcl-2 inhibits the mitochondrial changes including the reduction in $\Delta\psi_m$, but it fails to inhibit apoptosis and activation of caspases in certain cell systems (Scaffidi et al., 1998). Further evidence supporting this hypothesis comes from the studies using some specific genes knock-out mice.

Both Apaf-1 and caspase-9 are implicated in the initial activation of a caspase cascade (P. Li et al., 1997; Zou et al., 1997). Thymocytes from the Apaf-$1^{-/-}$ null-mutant mice are resistant to apoptotic cell death and maintain normal $\Delta\psi_m$ in response to a number of apoptotic stimuli including etoposide, dexamethasone, staurosporine and γ-irradiation (Yoshida et al., 1998). Thymocytes from the caspase-9 deficient mice also show resistance to apoptosis and retain their $\Delta\psi_m$ when treated with dexamethasone (Hakem et al., 1998). These studies demonstrate that the Apaf-$1^{-/-}$ and caspase-$9^{-/-}$ thymocytes are protected not only from apoptotic cell death but also from the mitochondrial changes, providing evidence that both Apaf-1 and caspase-9 lie upstream of the changes in $\Delta\psi_m$. Taken together, the results from the present study and studies of others support the idea that Apaf-1, in concert with cytochrome c and dATP, functions by activating caspases, which in turn cause the disruption in $\Delta\psi_m$.

Reduction in $\Delta\psi_m$ during the induction of apoptosis is believed to be mediated by the opening of the mitochondrial permeability transition (PT) pore (reviewed by Kroemer et al., 1997). However, to date the structure and composition of the PT pore still remain poorly defined, so does its physiological functions (reviewed by Green and Reed, 1998; Lemasters et al., 1998). It appears to be a complex of polypeptides located presumably at inner and outer membrane contact sites and possibly composed of both inner membrane proteins, such as the adenine nucleotide translocator (ANT), and outer membrane proteins, such as porin (Green and Reed, 1998). PT probably represents an abrupt increase of permeability of the mitochondrial inner membrane to solutes of molecular mass less than about 1.5 kDa (Zoratti and Szabo, 1995). Ca^{++}, P_i, and oxidant chemicals induce the opening of PT pore, whereas Mg^{++}, ADP, low pH and high membrane potential oppose the occurrence of PT. The rapid change in permeability resulting from the opening of PT pore causes loss of membrane potential, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, and mitochondrial swelling (Zoratti and Szabo, 1995). Recently, it has been reported that opening of PT pore does not occur exclusively during apoptosis and, in fact, it is a common mechanism for cell death by either necrosis or apoptosis (Lemasters et al., 1998). Therefore, these observations suggest that change in $\Delta\psi_m$ is not a key step in the apoptotic process. This, however, does not exclude the possibility that it may be critical for apoptosis in some cases. For

example, in apoptosis induced by some proapoptotic proteins such as BAX, mitochondrial changes including loss of $\Delta\psi_m$ are observed even when caspases are inhibited (Xiang et al., 1996).

7.2 Mechanisms regulating cell surface changes of cells undergoing apoptosis may not directly involve the activity of caspases

Apoptosis is a programmed form of cell death characterised by a series of biochemical and morphological changes affecting the nucleus, cytoplasm and plasma membrane. These changes in various cellular compartments are widely regarded as mechanistically linked events in a single "program", in which activation of caspases and proteolysis of intracellular substrates represent a final common pathway leading to cell death. In this study, using the mitochondrial inhibitors, antimycin A and oligomycin, the plasma membrane changes in cells undergoing apoptosis are dissociated from other features of the apoptotic phenotype, as described in chapter 4. In THP.1 cells triggered into apoptosis by etoposide or TPCK, these inhibitors blocked increased plasma membrane permeability, externalisation of PS and recognition by two classes of phagocytes utilising their respective receptors for PS and thrombospondin, but not activation of caspases-3 and -7, cleavage of PARP and DNA fragmentation. Similarly, externalisation of PS in apoptotic human leukaemic U937 cells is dissociated from activation of caspases. Z-VAD.FMK, on the other hand, inhibited all the above features of apoptosis. These findings could have at least two significant indications.

First, these data demonstrated for the first time that the surface changes of apoptosis relate to an independent pathway of events, which may be differentially regulated from the activation of "effector" caspases which are believed to trigger the nuclear and some of the cytoplasmic changes of apoptosis. Although these changes may occur simultaneously as part of a parallel process, mechanisms underlying these changes are potentially independent from each other. The effects of Z-VAD.FMK described here were in agreement with previous observations that activation of caspases is implicated in the externalisation of PS (Vanags et al., 1996; Martin et al., 1996). However, in the present study the "effector" caspases such as caspases-3 and -7 are clearly not involved, whereas in their studies they appear to be required. The

discrepancy in these findings may be reconciled by the observations that a loss of phospholipid asymmetry in the plasma membrane is often correlated with disruption or degradation of the cytomembrane network of spectrin, actin, and associated proteins (Williamson et al., 1987). The spectrin is capable of interacting directly with, in particular, the anionic phospholipids including PS that are concentrated in the inner leaflet of the membrane (reviewed by Schlegel and Williamson, 1987). Loss of the association with the proteins of the cytoskeleton results in an increased transbilayer movement of the aminophospholipids (Vermeulen et al., 1995). These results suggest that the membrane skeleton proteins are not only responsible for maintaining cell shape but also upholding the membrane phospholipid asymmetry. During the induction of apoptosis, cleavage of a subset of the membrane skeleton proteins was observed, including actin (Mashima et al., 1995; Kayalar et al., 1996; Brown et al., 1997), fodrin (non-erythroid spectrin) (Martin et al., 1995a; Cryns et al., 1996; Vanags et al., 1996) and Gas2 protein, a component of the microfilament system (Brancolini et al., 1995). Most, if not all, of these proteins are cleaved by various members of caspases. Thus it appears likely that the involvement of caspases in the PS exposure in apoptosis is probably through the proteolysis of some of these membrane skeleton proteins, which in turn result in a loss of phospholipid asymmetry in the membrane, leading to PS exposure. Future work will need to determine whether the two mitochondrial inhibitors block the cleavage of these proteins, which may shed light to the understanding of the mechanisms of their inhibition on the membrane changes including PS exposure during the induction of apoptosis. In addition, it has been reported that expression of Bcl-2 in neutrophils inhibits their apoptosis as assessed by a loss of viability and internucleosomal DNA fragmentation, but not engulfment by macrophages (Lagasse and Weissman, 1994). This suggests that mechanism(s) triggering phagocytosis of aging neutrophils is independent from the process of apoptosis regulated by Bcl-2. It will be interesting to see whether activation of caspases is also inhibited in these neutrophils, thus further testing the hypothesis of caspase-independent pathway(s) of cell surface changes leading to recognition and phagocytosis.

Secondly, this finding of dissociation could have very important consequences for attempts to manipulate apoptosis for therapeutic gain. In disorders characterised by

unscheduled cell loss by apoptosis, such as neurodegenerative disease, potential therapies will not only need to block intracellular pathways leading to engagement of effector enzymes but will also need to interfere with those governing recognition, in case potentially rescued cells are undesirably removed by phagocytes. Conversely, there may be potential for selective triggering of the surface changes of apoptosis in "undesirable" cells (such as cancer cells) so that these are removed by innate mechanisms.

It is of interest that the dissociation of the cell surface changes from the other changes of the apoptotic phenotype was accomplished using antimycin A and oligomycin, two agents which interfere with mitochondrial functions. The blockade of this putative 'recognition pathway' by the two mitochondrial inhibitors provides important clues for its further dissection. A critical role for the mitochondrial release of proteins, such as apoptosis inducing factor (AIF) and cytochrome c, has been proposed in initiating the apoptotic program including DNA fragmentation and activation of caspase-3 (Susin et al., 1996a; Liu et al., 1996). Bcl-2 may prevent apoptosis in part by blocking the mitochondrial release of both AIF and/or cytochrome c (Susin et al., 1996a; Yang et al., 1997; Kluck et al., 1997). Although antimycin A and oligomycin may affect targets other than their known mitochondrial ones, it is possible that two mitochondrial inhibitors may have interfered with mitochondrial function, so preventing the mitochondrial release of some factor(s), other than cytochrome c or AIF, which directly or indirectly results in exposure of PS.

In addition, the finding of the dissociation of the cell surface changes including PS exposure from the other changes of the apoptotic phenotype appears to be analogous to a clinical condition known as Scott syndrome. Scott syndrome is a rare inherited bleeding disorder characterised by the inability of blood platelets and other cells including erythrocytes to expose PS in response to activation by Ca^{++}, resulting in deficient procoagulant activity (Sims et al., 1989; Weiss, 1994). This disorder is generally believed to be related to a defective gene that selectively affects membrane phospholipid scrambling (Bevers et al., 1992; Toti et al., 1996). Recent studies show, however, that the scramblase isolated from detergent-solubilised Scott erythrocytes exhibits normal phospholipid scrambling activity (Stout et al., 1997). Scott

lymphoblasts also expressed normal levels of phospholipid scramblase mRNA and protein (Zhou et al., 1998). These results suggest that the defect in Scott syndrome is related to an aberrant posttranscriptional processing of the scramblase polypeptide preventing its interaction with Ca^{++} *in situ*, or alternatively, due to the presence of a detergent-dissociable inhibitor in the Scott cells. If true, it may be possible that the mitochondrial inhibitors either interact with the enzyme or enhance the binding of the inhibitor to it, thus blocking the access of Ca^{++} to its binding sites in phospholipid scramblase. There may also be the possibility that the mitochondrial inhibitors block the upstream signal(s) leading to the activation of the scramblase.

7.3 Cytochrome c release represents the commitment point of cell death in chemical-induced apoptosis

The data obtained from this study strongly support the view that caspases are the major biochemical execution machinery during the induction of the apoptosis by etoposide or TPCK. Activation of caspases occurred early with the subsequent cleavage of intracellular proteins such as PARP and lamins, which precipitated the dramatic morphological and other biochemical changes of apoptosis. Inhibition of caspase activation by the tripeptide inhibitor, Z-VAD.FMK, blocked all the changes characteristic of apoptosis. This appears to indicate that activation of caspases may be an irreversible commitment of cells to death in chemical-induced apoptosis. However, two pieces of evidence from the present study argue against this proposition. First, Z-VAD.FMK did not block the release of cytochrome c during the induction of apoptosis by either etoposide or TPCK in THP.1 cells, as described in chapter 5, (Fig. 16 A and B lanes 7), nor did it block this release during the induction of apoptosis by etoposide in U937 cells (Fig. 19 D lane 5) although it blocked that in TNF-α-mediated apoptosis (Fig. 19 D lane 3). Secondly and more importantly, in THP.1 cells incubated with etoposide in the presence of Z-VAD.FMK for 24 h, over one third of total cell population lost their viability, as assessed by trypan blue dye inclusion even though the activation of caspases was still inhibited (Fig. 22 lane 6 in chapter 6). The latter observation suggests that these cells are not fully protected from subsequent cell death even when caspases are inactivated. It further implies that cells become irreversibly committed to cell death before the activation of caspases following treatment with etoposide. This notion is compatible with an earlier

observation that inhibition of caspases does not prevent cell death induced by oncogenes , DNA damage and Bak, a proapoptotic member of the Bcl-2 family in rat fibroblasts (McCarthy et al., 1997). In this study, it is also shown that cells treated with various apoptotic stimuli in the presence of Z-VAD.FMK have no clonogenic potential and can not be rescued by the addition of antiapoptotic survival factors. Another recent study show that Jurkat cells co-incubated with etoposide or actinomycin D or staurosporine and Z-VAD.FMK are incapable of proliferating as determined by lack of clonogenic potential and they subsequently die by necrosis (Amarante-Mendes et al., 1998). These studies suggest that activation of caspases follows an event that commits cells to die under these circumstances.

There is an increasing evidence suggesting that release of mitochondrial cytochrome c represents the commitment point of cell death in chemical-induced apoptosis. In the present study, while a direct temporal relationship between cytochrome c release and activation of caspases had not been established in intact cells, release of cytochrome c was observed as an early event during the induction of apoptosis by etoposide or TPCK, occurring prior to both the reduction in $\Delta\Psi_m$ and PS exposure (Fig. 16 in chapter 5). The observation that Z-VAD.FMK did not block the release of cytochrome c in chemical-induced apoptosis in both THP.1 and U937 cells demonstrated that this release was upstream of the activation of caspases. Cytochrome c has been shown to complex with Apaf-1, in the presence of dATP, activating caspase-9, so initiating the caspase cascade (Liu et al., 1996; P. Li et al. 1997; Zou et al., 1997). These results suggest that release of cytochrome c is a critical event, involved in triggering caspase activation which subsequently brings about the caspase-dependent apoptotic changes. This notion is supported by many observations of other studies in the literature. Release of cytochrome c is observed in apoptosis induced by a variety of stimuli including UV irradiation, staurosporine, actinomycin D, etoposide, ara-C, hydrogen peroxide, and ligation of the death receptor (e.g. CD 95) (Krippner et al., 1996; Liu et al., 1996; C. Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997; Bossy-Wetzel et al., 1998; Scaffidi et al., 1998; Vander Heiden et al., 1998). Immunocytochemistry confirms the translocation of cytochrome c from the mitochondria to the cytoplasm following stimulation with UV irradiation in HeLa cells (Bossy-Wetzel et al., 1998) and in embryonic stem cells (Hakem et al., 1998) or

with etoposide, staurosporine and UV irradiation in embryonic fibroblast cells (Yoshida et al., 1998). These results suggest that the release of cytochrome c is a common event in apoptosis, which acts as a converging point from diverse apoptotic signal-transducing pathways. As Z-VAD.FMK inhibits cytochrome c release and cell death in CD95 receptor-mediated apoptotsis in Jurkat cells (Vander Heiden et al., 1998; Amarante-Mendes et al., 1998) and protects cells against CD95-induced apoptosis in mice (Rodriguez et al., 1996; Chandler et al., 1998), it appears that the commitment to cell death is caspase-dependent in death receptor-mediated apoptosis. Release of cytochrome c is thus not an essential event in these cases. However, Z-VAD.FMK does not block cytochrome c release in chemical-induced apoptosis, as described here, and in apoptosis induced by UV irradiation or staurosporine in human T lymphoblastoid CEM cells (Kluck et al., 1997; Bossy-Wetzel et al., 1998) and in Jurkat cells (Vander Heiden et al., 1998). These results suggest that Z-VAD.FMK does not inhibit the commitment to cell death but rather inhibits all the biochemical changes associated with caspase activation. Further evidence is provided by several gene knock-out studies. Cytochrome c has been shown to interact with Apaf-1, activating caspase-9, which in turn activates the effector caspases such as caspase-3 (Liu et al., 1996; P. Li et al. 1997; Zou et al., 1997), suggesting that Apaf-1 and caspase-9 are the important components in cytochrome c-mediated caspase-activating pathways. Thymocytes from caspase-9 deficient mice are resistant to cell death induced by many different stimuli including dexamethasone, etoposide and γ irradiation, but not to apoptosis by engaging the death receptors with CD95 and TNF-α (Hakem et al., 1998; Kuida et al., 1998). In Apaf-$1^{-/-}$ thymocytes and embryonic fibroblasts, resistance to cell death induced by apoptotic stimuli such as etoposide, dexamethasone and staurosporine is observed, but not in CD95-induced apoptosis (Cecconi et al., 1998; Yoshida et al., 1998). These results demonstrate that Apaf-1 and caspase-9 are required in the induction of apoptosis by a mechanism other than the death receptor-mediated one. Because the release of cytochrome c is observed during the induction of cell death in both caspase-$9^{-/-}$ cells (Hakem et al., 1998) and Apaf-$1^{-/-}$ cells (Yoshida et al., 1998), it suggests that both Apaf-1 and caspase-9 act downstream of cytochrome c release. Thus, taken together, these results clearly demonstrate that release of cytochrome c is a central event in chemical-induced apoptosis, involved in the commitment of cells to die. Caspase activation occurs after

commitment to cell death and is primarily responsible for those stereotypic biochemical and morphological changes commonly associated with the apoptotic phenotype. In this model of apoptosis, therefore, caspases act solely as the executioners. Meanwhile, Z-VAD.FMK inhibits apoptosis downstream of cell death commitment by inhibiting the initiator caspase-9 and the resultant postmitochondrial activation of effector caspases. This interpretation may have some clinical implications for the use of caspase inhibitors as potential therapeutic agents. Cells, which are committed to die but with caspases inhibited, may die more slowly and with a different morphology from cells dying with characteristic apoptotic morphology, as demonstrated by recent reports describing a caspase-independent cell death, such as Bax- or Bak-induced cell death (Xiang et al., 1996; McCarthy et al., 1997).

7.4 Molecular ordering of the cell death pathways in chemical-induced apoptosis

A model of apoptosis in which the release of cytochrome c represents the commitment point of cell death is proposed (Fig. 26). Diverse cell death-transducing signals converge on mitochondria to induce the release of cytochrome c. The mechanisms of this release in chemical-induced apoptosis is not yet clear. Cytochrome c release has been reported to be prevented by anti-apoptotic proteins, Bcl-2 or Bcl-x_L (C. Kim et al., 1997; Kluck et al., 1997; Yang et al.,1997; Vender Heiden et al. 1997; Bossy-Wetzel, 1998). Once released, in the presence of dATP, cytochrome c interacts with apoptotic protease activating factor-1 (Apaf-1), resulting in activation of caspase-9, which in turn activates downstream effector caspases such as caspase-3 (Liu et al., 1996; P. Li et al. 1997; Zou et al., 1997). Activated caspases then cleave a subset of important intracellular protein substrates, which are believed to be associated with the stereotypic biochemical and morphological changes of the apoptotic phenotype. Z-VAD.FMK inhibits the activation of the initiator caspase-9 and the effector caspases. Caspases can also act on mitochondria to induce an opening of the mitochondrial PT pore. As a result, $\Delta\Psi_m$ becomes dissipated with subsequent generation of reactive oxygen species (ROS) and release of apoptosis-inducing factor (AIF) from mitochondria, which further accelerate the death process. The plasma membrane changes such as PS exposure in apoptotic cells serve as signals for phagocytic recognition and clearance of the dying cells (Savill et al., 1993; Pradhan et al., 1997;

Savill, 1998). These membrane changes may be inhibited by the mitochondrial inhibitors antimycin A and oligomycin by a yet unknown mechanism(s).

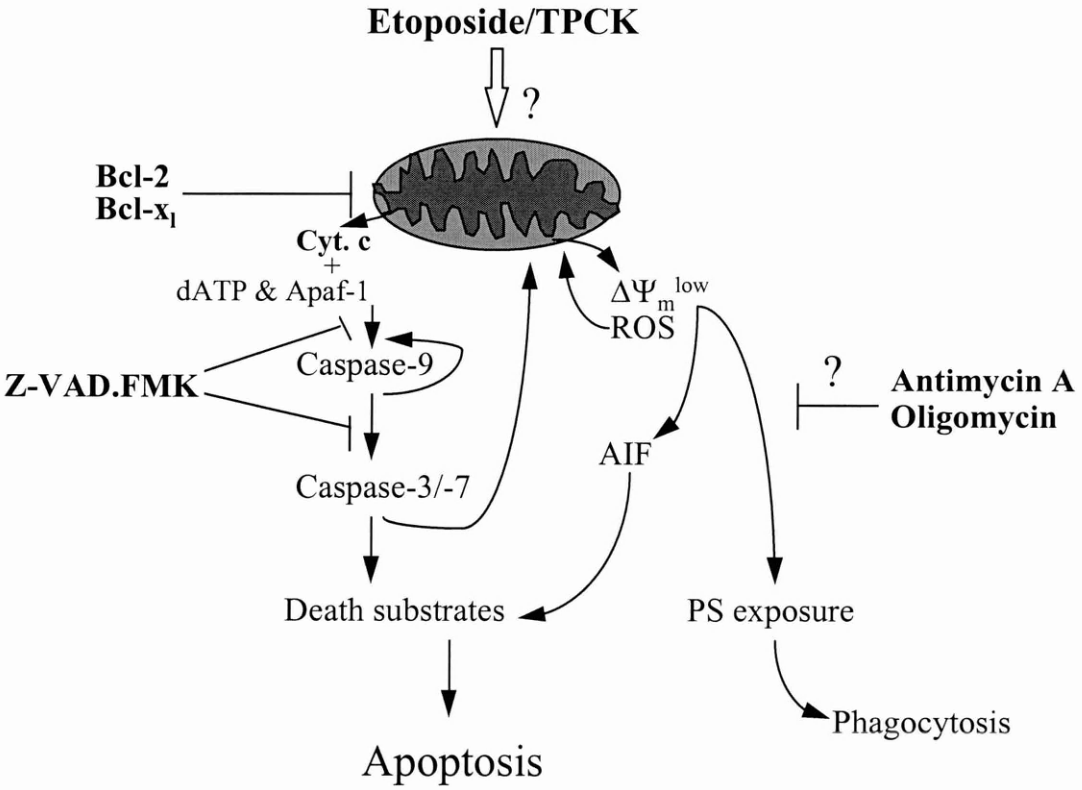


Figure 26. Schematic diagram of the caspase activation pathways in chemical-induced apoptosis

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